USING MOLECULAR GENETIC TECHNIQUES TO DETECT OUTCROSSING IN NATURAL POPULATIONS OF A SELF-FERTILIZING FISH

by

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Using molecular genetic techniques to detect outcrossing in natural populations of a self-fertilizing fish.

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(ABSTRACT)

The hermaphroditic fish, *Rivulus marmoratus*, is the only vertebrate known to reproduce by internal self-fertilization; this process results in populations of homozygous clones. Most natural populations consist entirely of hermaphrodites, but phenotypically distinct, fertile males occur at frequencies up to 24% on some islands off the coast of Belize. The presence of large numbers of males in natural populations prompted this study to determine if males are involved in the mating system. The occurrence of cross-fertilization between males and hermaphrodites was determined by surveying progeny of field-caught hermaphrodites for non-segregation or segregation of DNA fingerprint markers as an indication of the homozygosity or heterozygosity of the parent.

DNA fingerprinting revealed no segregation of markers among the offspring in 12 of 12 Florida and Brazil laboratory lines and in 5 of 30 Belize Cay broods. These data indicate that the hermaphrodite parents were homozygous; thus, no detectable outcrossing has occurred in these populations. However, DNA fingerprinting revealed segregation of markers among the offspring in 25 of 30 Belize Cay broods. Twenty-four of these broods were from the island of Twin Cays. An average of 30% of the parental bands were segregating among the offspring; values ranged from 0.09 to 0.50. Offspring were, on average, 8% dissimilar to one another; values ranged from 2.08% to 15.09%. These data suggest that the 25 hermaphrodite parents were heterozygous; thus, males are involved in the mating system in some Belize Cay populations. These data are the first evidence of outcrossing in this species.

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INTRODUCTION

The majority of animal species, including most vertebrates, reproduce sexually. Sexual reproduction results in variable progeny due to segregation and recombination events during meiosis. Though the precise advantage has been difficult to identify (Hamilton et. al., 1990), sexual reproduction is widely considered advantageous (Williams, 1975; Maynard Smith 1978; Bell, 1982). Populations with recombination can evolve faster by natural selection than those without recombination. This is because sexual populations can accumulate beneficial gene combinations at high rates, and only accumulate harmful mutations at low rates (since offspring can be produced lacking the mutation) (Muller, 1932, 1964; Crow and Kimura, 1965; Pamilo et. al., 1987). Populations with recombination can adjust more quickly to changing abiotic and biotic environments than those without recombination. If the environment changes temporally, sexual populations can respond quickly when the direction of selection changes and thus maintain fitness (Van Valen, 1973; Hamilton, 1980; Bell 1982). If the environment changes spatially, variable offspring can exploit a wide variety of niches (Maynard Smith, 1978; Bell, 1982). Finally, if the environment changes unpredictably in time or space, variable offspring are more likely to be advantageous (Williams, 1975). These assertions are supported by the dominance of sexually reproducing organisms.

Approximately 50 species of vertebrates (among 22 genera of fishes, amphibians, and reptiles) reproduce by mechanisms lacking recombination (Vrijenhoek et. al., 1989) resulting in the production of genetically identical progeny (i.e., identical to one another and to the parent). The existence of these organisms, although they represent only 0.1% of the total number of vertebrate species (Vrijenhoek, 1989), suggests that this mode of reproduction may be advantageous under some circumstances. By means of clonal reproduction, organisms can efficiently transmit favorable gene

combinations from one generation to the next. Further, these organisms can always be certain of reproductive success (Tomlinson, 1966). They can save time and energy by not mating, and can more easily colonize new habitats (Hughes, 1989). However, because of genetic, developmental, and ecological constraints, clonal organisms are considered to have limited evolutionary potential (Maynard Smith, 1978; Vrijenhoek, 1989). This is supported by the rarity of clonal vertebrates. Populations without recombination cannot evolve as fast under the influence of natural selection as populations with recombination. Clonal reproduction, thus, is apparently disadvantageous because clonal lineages will accumulate harmful mutations at high rates (since all offspring will inherit all mutations of the parent resulting in a continual increase of mutations within a clonal line) and accumulate beneficial gene combinations at low rates (since different beneficial mutations must occur within the same individual) (Fisher, 1930; Muller, 1932, 1964; Felsenstein, 1974). The lack of recombination prevents adjustment to changing abiotic environments as specialized clones may be eliminated by environmental change (Vrijenhoek, 1989). Clonal lines are more prone to higher extinction rates (Maynard Smith, 1978), lower diversification rates (Stanley, 1975), and the inability to prevent the accumulation of deleterious mutations which reduce fitness (Muller, 1964; Shields, 1982). However, this lack of evolutionary potential has recently been questioned due to the discovery of high levels of clonal diversity in natural populations and the success of some clonal vertebrates (Angus, 1980; Dawley, 1989; Dessauer and Cole, 1989; Parker et. al., 1989; Turner et. al., 1990). Clonal diversity in some species is probably due to high levels of heterozygosity resulting from multiple hybridization founding events and mutation (Dawley, 1989; Parker et. al., 1989). Thus, individual clonal lines may represent dead ends, but clonal populations may persist if new clones continually arise from independent origins (such as recurrent hybridization events) (Vrijenhoek, 1989).

The hermaphroditic fish, *Rivulus marmoratus*, is the only vertebrate known to reproduce by internal self-fertilization (Harrington, 1961, 1963). Most natural populations of *R. marmoratus* consist entirely of internal self-fertilizing hermaphrodites which resemble females of other species of *Rivulus* (Huber, 1992). No true females are known. Phenotypically distinct fertile males were surprisingly discovered initially in laboratory populations (Harrington, 1961) and later in natural populations (Kristensen, 1970; Davis et. al., 1990). Males can be induced in the laboratory by manipulations of environmental factors (temperature and photoperiod) (Harrington, 1967, 1968, 1971, 1975). In Florida populations, males occur at extremely low frequencies (<1%). However, on some barrier islands off the coast of Belize, males occur at unusually high frequencies (13.5-24%) (Davis et al., 1990; Turner et. al., 1992a). Neither the production of males within clonal lines in laboratory populations nor the abundance of males in a few natural populations is understood. The occurrence of males suggests differences among natural populations in the stability of the sex determination system (Harrington, 1967), and also suggests the possibility of outcrossing in this species (Harrington, 1961; Atz, 1965; Harrington, 1967; Kristensen, 1970; Harrington, 1971; Vrijenhoek, 1985; Hughes, 1989).

Self-fertilization is the ultimate form of inbreeding. Heterozygosity is reduced by half each generation and thus selfing should lead to homozygosity within ten generations (Haldane, 1936). Populations of *R. marmoratus* do consist of homozygous clones (isogenic uniparental lines) (Kallman and Harrington, 1964; Harrington and Kallman, 1968; Turner et. al., 1990). In contrast, most other clonal vertebrates are heterozygous, not homozygous (Dawley, 1989; Parker et. al., 1989). There is no evidence that *R. marmoratus* is of hybrid origin, so the only source of variation within clonal lines is mutation (Turner et. al., 1991, 1992b). In theory, *R. marmoratus* has limited evolutionary potential, yet it has colonized

highly variable mangrove forests and has achieved a widespread geographic distribution.

Are males involved in sexual reproduction in natural populations of *R. marmoratus*? Such a role would provide a mechanism by which recombination could be introduced into an otherwise clonal organism. Males appear sexually competent. Both laboratory-induced males and field-caught males have mature sperm (Harrington, 1975; Turner et. al., 1992a). Spawning embraces and courtship displays have been observed in the laboratory between males and hermaphrodites (Harrington, 1971; Davis et. al., 1990; Turner et. al., 1992a). However, fertilization is virtually synonymous with ovulation; most eggs are oviposited within 24 hours of fertilization but can be incubated for as long as 3 days before oviposition (Harrington, 1963, 1967). Thus, the potential for cross-fertilization exists only when a hermaphrodite lays infertile eggs. This may occur on very rare occasions when the testicular and ovarian components of the ovotestis become out of phase (Harrington, 1963).

The objective of this study was to determine the role of males in natural populations of *Rivulus marmoratus* from the Belize barrier islands. The presence of large numbers of males in natural populations on the Belize Cays directly prompted this study. The ability to investigate this mating system has only recently become possible due to the discovery of high levels of variation at the molecular level in *R. marmoratus*. The conventional population genetic technique of allozyme electrophoresis (the characterization of allelic forms of enzymes produced by specific gene loci) (Harris, 1966; Lewontin and Hubby, 1966) detected little variation in natural populations of *R. marmoratus* (Massaro et. al., 1975; Vrijenhoek, 1985). By contrast, the molecular technique of multilocus DNA fingerprinting (the detection of hypervariable repetitive DNA sequences) (Jeffreys et. al., 1985; Tautz, 1989; Epplen et. al., 1991) detected high levels of clonal diversity in natural populations of *R.*

marmoratus (Turner et. al., 1990, 1991, 1992b). DNA fingerprinting was used in this study to provide potentially polymorphic genetic markers for the assessment of heterozygosity in field-caught hermaphrodites from the Belize Cays; laboratory-reared progeny of these hermaphrodites were surveyed for segregating DNA fingerprint markers. This study presents evidence for a novel mating system in the self-fertilizing hermaphroditic fish, *Rivulus marmoratus*, involving cross-fertilization between males (presumably environmentally-induced) and hermaphrodites, thus generating genetically variable offspring.

MATERIALS AND METHODS

STUDY ORGANISM

Distribution and natural history

Rivulus marmoratus is distributed in marine and semiterrestrial habitats from Florida, U.S.A. throughout the Caribbean to Southeastern Brazil and possibly to Argentina (Huber, 1992). Of the 70 well-defined species in the genus *Rivulus* (family Cyprinodontidae, subfamily Rivulinae) (Huber, 1992), *R. marmoratus* is the only known hermaphroditic species. *R. marmoratus* was first described from Cuba by Poey in 1880 but was considered a synonym of *R. cylindraceus*, another Cuban species, and not recognized as a distinct species until Rivas (1945). The species was initially discovered in Florida by Harrington and Rivas (1958). Though usually found in marine or brackish water, *R. marmoratus* can also be found in fresh water (Harrington, 1961; Kristensen, 1970; Huber, 1992) and even out of water (Huehner et. al., 1985; Abel, 1987). The neotropical fish genus, *Rivulus*, is renowned for the unusual behavioral habit of spending lengthy periods of time out of the water (Huber, 1992). Because *R. marmoratus* is found in Western Atlantic mangrove habitats, Davis et. al. (1990) call this species the mangrove rivulus. Though once thought to be rare, recent studies have shown that *R. marmoratus* is actually quite abundant in a specific microhabitat (land crab burrows) in the highly variable mangrove forests and salt marshes (Davis et. al., 1990).

Taxonomic note

R. marmoratus is listed (as well as *R. bonairensis*) as a junior synonym of *R. ocellatus* (Huber, 1992), but the species designation is currently in review. A petition was filed by Lazara and Smith (1990) to the International Commission on Zoological Nomenclature to reject

the senior synonym *ocellatus* as invalid and to designate *marmoratus* as the valid name. *R. ocellatus* is a poorly known species and has been misidentified on occasion as another species, *R. caudomarginatus* (Lazara and Smith, 1990), the only other *Rivulus* species found in brackish water (Huber, 1992).

Sampling methods and locations

Laboratory-reared parents and offspring of specimens originally collected in Florida, U.S.A. and Rio de Janeiro, Brazil were used in this study as well as field-caught specimens from Belize, Central America and their laboratory-reared offspring (Figure 1). All original field-caught adult specimens were collected in mangrove swamps or salt marshes from leaf litter, land crab burrows, and other depressions filled with water. Fish were collected using light tackle, dip nets, and funnel traps (Taylor, 1988).

Sampling locations in Florida of the field-caught progenitors of laboratory lines (n=11 lines) included Vero Beach (n=2), Miami (n=1), Marco Island/Rookery Bay (n=3), Everglades National Park (n=3), and No Name Key (n=2) (Figure 2). These lines consisted of both longer-term lines (collected before 1986) and shorter-term lines (collected in 1989 and 1990).

The sampling location in Brazil of the field-caught progenitor of a single laboratory line (n=1) was near Rio de Janeiro in January, 1989.

Sampling locations in Belize of the field-caught hermaphrodites included 4 mangrove islands adjacent to the Belize barrier reef. Thirty specimens were collected in January 1991 on Coco Plum Cay (n=4 fish), Ragged Cay (n=1), Twin Cays (n=24), and Pelican Cay (n=1). The islands are located at approximately Lat. 16°53' N, Long. 88°7' W, Lat. 16°51' N, Long. 88°8' W, Lat. 16°50' N, Long. 88°6' W, Lat. 16°41' N, Long. 88°10' W for Coco Plum Cay, Ragged Cay, Twin Cays, and Pelican Cay respectively (Figure 3). Field collection data

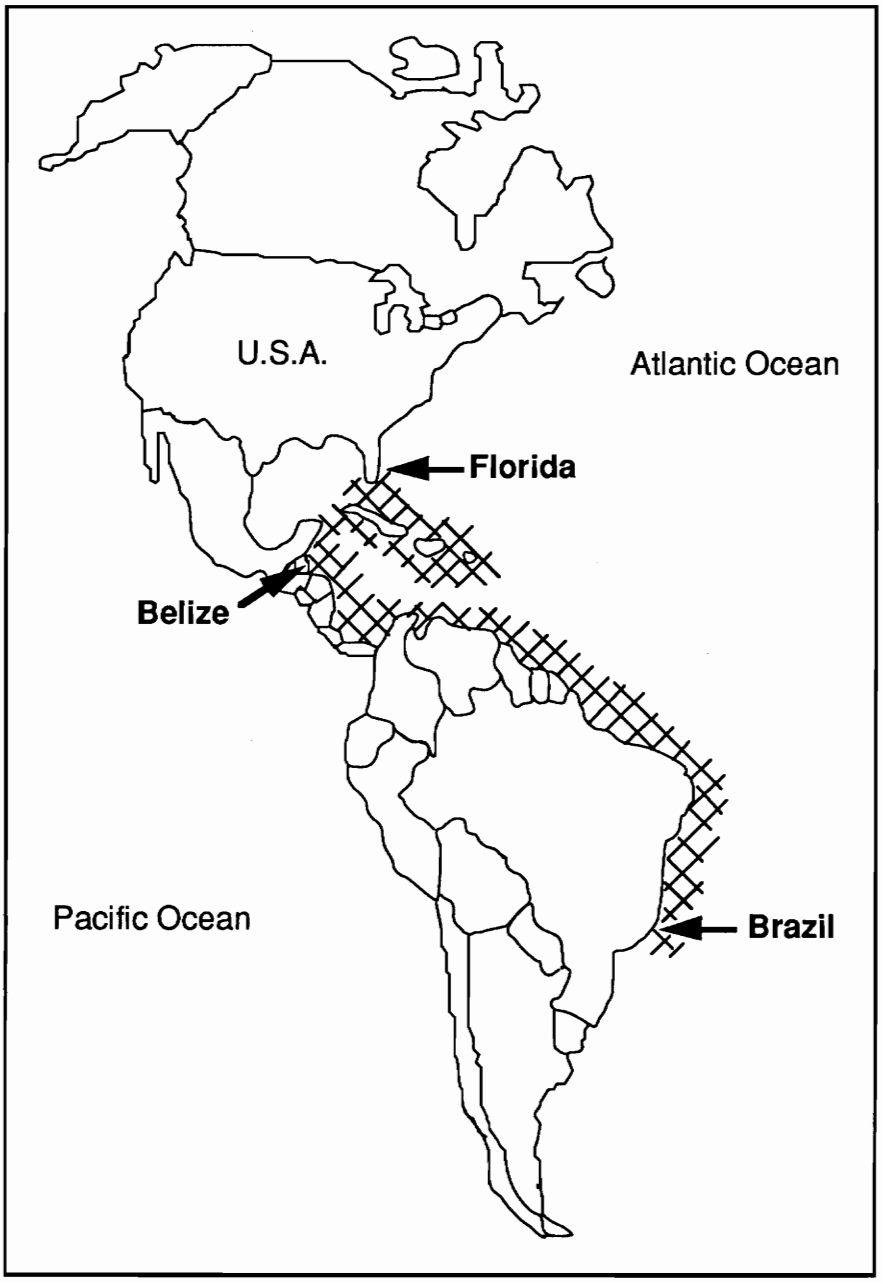


Figure 1. Map illustrating the geographic range and the general collecting locations of specimens of *Rivulus marmoratus*. The geographic range of *R. marmoratus* is indicated by the hatched lines. Collecting locations included Florida at the northern end of the range, Brazil at the southern end, and Belize in the middle of the range.

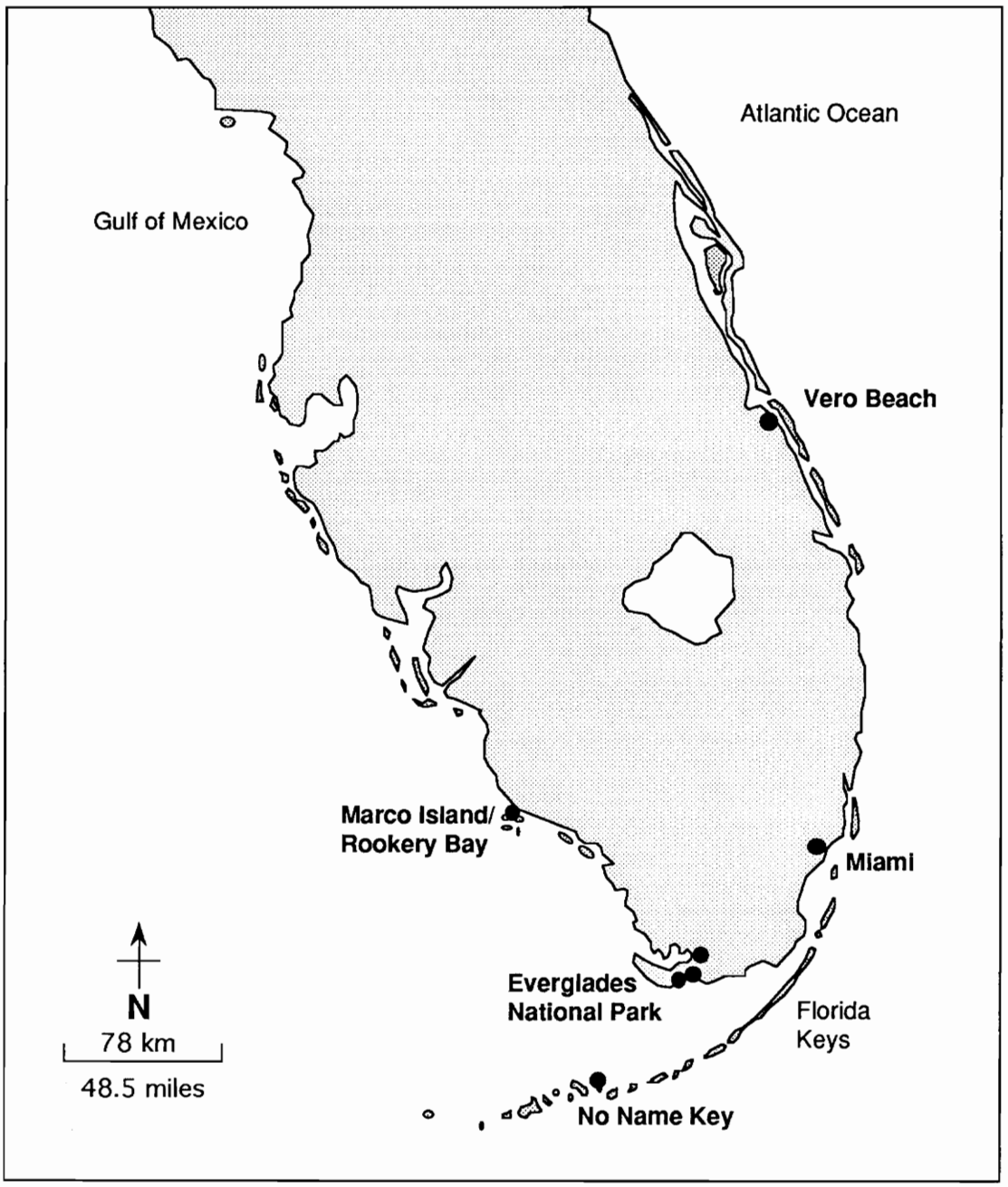


Figure 2. Map of collecting locations of progenitors of Florida laboratory lines. Locations included: Vero Beach (n = 2 lines), Miami (n = 1 line), Marco Island/ Rookery Bay (n = 3 lines), Everglades National Park (n = 3 lines), and No Name Key (n = 2 lines).

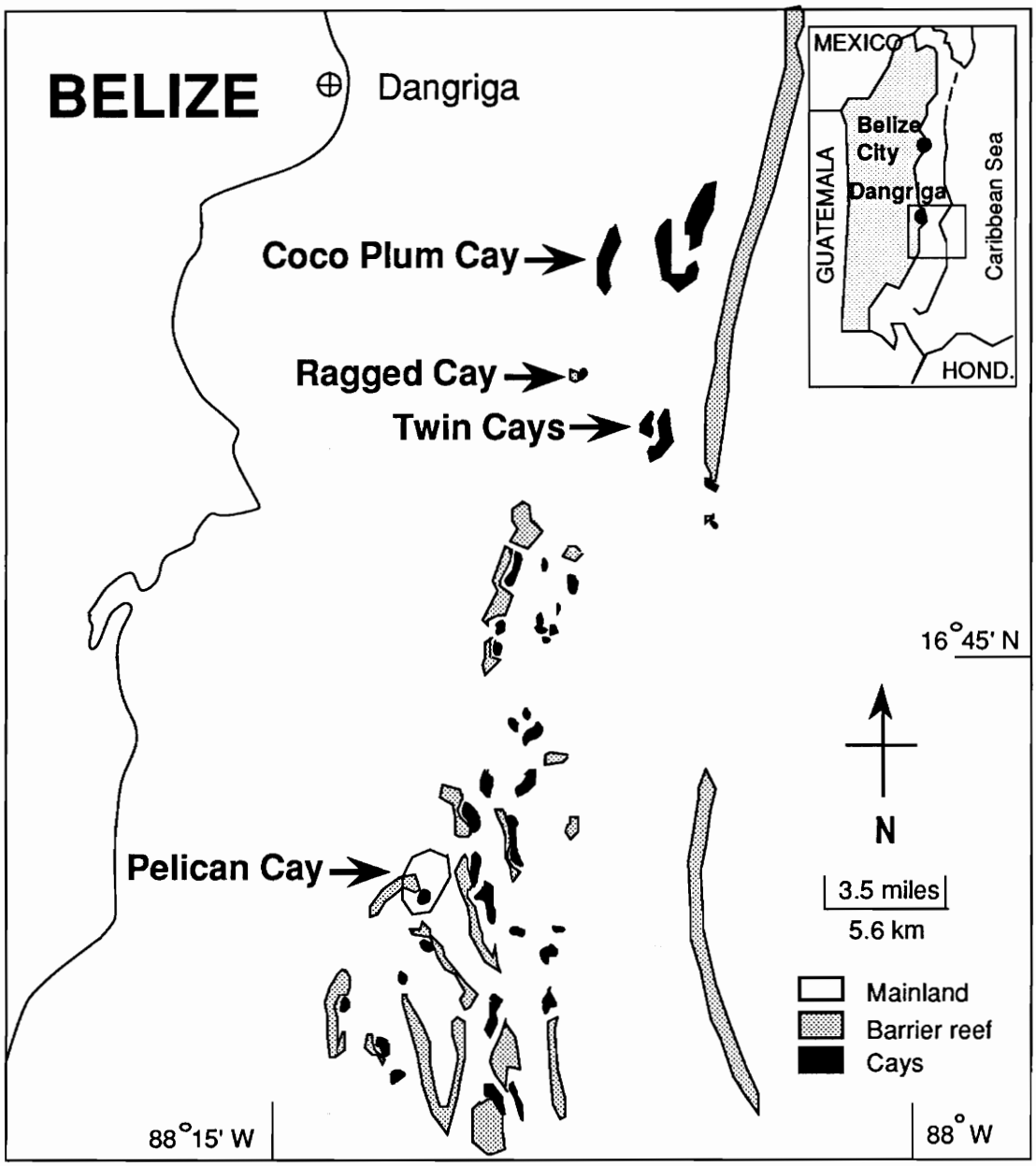


Figure 3. Map of mangrove islands adjacent to the Belize barrier Specimens were collected on Coco Plum Cay (n = 4 fish), Ragged Cay (n = 1 fish), Twin Cays (n = 24 fish), and Pelican Cay (n = 1 fish).

from these 4 islands is shown in Table 1.

Lab breeding and rearing

Field-caught specimens, laboratory-raised specimens, and all their offspring were maintained in the laboratory under constant photoperiod conditions (16 hours Light, 8 hours Dark) at a salinity of approximately 30% (wt./vol.) artificial sea water (7-8 ppt) made from Instant Ocean (Aquarium Systems, Mentor, OH) powder. Hermaphrodites (both laboratory line specimens and Belize Cay specimens) were housed individually in 10.5 cm glass fingerbowls containing a 10 cm mesh screen (with holes 1mm in width) on the bottom to facilitate egg collection. Eggs were collected from each adult and kept in petri plates with 30% (wt./vol.) artificial sea water until hatching. The numbers of eggs laid and hatching dates of offspring were recorded for each parent.

Upon hatching, the fry were moved to new containers; offspring from the same parent were kept together as long as the fry hatched within 7 days of one another. The hatchlings were raised for a minimum of 3 months until each was of the appropriate size for DNA fingerprinting (at least 10-11 mm standard length). The sex (hermaphrodite or male) of each hatchling was also determined. Fish reach sexual maturity at approximately 3-6 months of age (Harrington, 1967; Huber, 1992). Hermaphrodites and males raised at the same temperatures become recognizable at approximately the same size and age (Harrington, 1967). Fish were scored as hermaphrodites if there was a distinct caudal ocellus (a juvenile trait retained in hermaphrodites) and no sign of orange coloration on the body or fins; fish were scored as males if orange coloration was present on the body or fins. In males, the caudal ocellus is generally faded or lacking; also, the ventral margin of the caudal fin may be black (Harrington, 1967; Harrington, 1968).

Table 1. Field collection data of *R. marmoratus* from the Belize barrier islands of Pelican Cay, Ragged Cay, Coco Plum Cay, and Twin Cays. Reported numbers are the number of males collected / total number of fish collected on each island.

BELIZE CAYS	#males/total	year	% of males
Pelican Cay	0/3	1991 *	<33%
Ragged Cay	0/40	1991 *	<2.5%
Coco Plum Cay	0/28	1991 *	<3.6%
Twin Cays	32/134	1988 @	23.9%
	21/148	1989 @	14.2%
	11/159	1991 *	6.9%
	16/254	1992 *	6.3%
Twin Cays total	80/695	1988-1992	11.5%

references:

* D.S. Taylor, personal communication

@ Turner et. al., 1992a

DNA FINGERPRINTING

DNA isolation

To obtain high molecular weight nuclear DNA, the procedures of Turner et. al. (1989) and Sambrook et. al. (1989) were modified. To isolate DNA from fry, whole fish (not fed 24 hours prior to sacrifice) were minced with a razor blade and placed into a test tube containing 2 ml of extraction buffer and 200 ug of proteinase K (Gibco BRL, Gaithersburg, MD). Extraction buffer contained 10 mM Tris, 0.1 M (Ethylenedinitrilo)-Tetraacetic acid Disodium Salt (EDTA), and 0.5% Sarkosyl (Fisher Scientific, Fair Lawn, NJ) (pH 8.0). Samples were incubated overnight in a 50 °C water bath. No homogenization was necessary. An equal volume of phenol:chloroform equilibrated with 1 X TE (10 mM Tris, 1 mM EDTA) was added to the homogenate, mixed gently to avoid shearing the DNA, and centrifuged. The supernatant was drawn off with a wide bore transfer pipette and used in the next extraction. Samples were extracted twice with an equal volume of 1:1 phenol:chloroform and once with an equal volume of 24:1 chloroform:isoamyl alcohol. Between each extraction, the samples were centrifuged at 10,000 x g for 30 minutes at room temperature. No precipitation step was used. The final supernatant (DNA sample) was dialyzed against 1 X TE for at least 48 hours at 4 °C to get rid of the proteinase K and low molecular weight contaminants. For dialysis, DNA samples (2 ml) were placed into a 120 mm (in length) piece of 10 mm flat width Spectra/Por Molecular Porous Membrane, MWLO 12-14,000 (Spectrum Medical Industries, Inc., Los Angeles, CA). The TE was replaced every 24 hours.

To isolate DNA from adults, whole degutted fish (not fed 24 hours prior to sacrifice) were minced and incubated overnight as above in 4 ml of extraction buffer containing 400 ug of proteinase K. Samples were extracted as above, precipitated with 0.2X volume 10 M ammonium acetate and two volumes cold 95% ethanol overnight at -20 °C. The DNA was recovered by centrifugation at 10,000 x g for

one hour at -20 °C. DNA pellets were washed with 70% ethanol, dried, and redissolved in 1 ml 1 X TE overnight at 37°C. Samples were dialyzed as above in pieces of dialysis tubing 75 mm in length.

Measurement of DNA concentration

A DNA fluorometer, TKO 100 Mini Fluorometer (Hoefer Scientific Instruments, San Francisco, CA), was used as recommended to measure the DNA concentration of the samples. Salmon sperm DNA (250 ug/ml) was used as a reference standard. A working dye solution was prepared consisting of 0.1 ug/ml Hoechst dye 33258 (Polysciences Inc., Warrington, PA) in 1 X TNE (10 mM Tris, 1 mM EDTA, 0.1 M NaCl, pH 7.4). Four ul of sample were placed into a cuvette containing 2 ml of working dye solution. The cuvette was inverted continuously for 1 minute before reading and rinsed with distilled water between samples.

DNA yields of fry (approximately 11-30 mm standard length) were generally between 30 ug and 200 ug per fish. DNA yields from adults (approximately 28-50 mm standard length) were generally between 270 ug and 500 ug per fish.

DNA was visualized on a 1% agarose gel to ensure high molecular weight material was obtained. Five ul of sample and 3 ul of loading buffer containing 0.2 ug/ul ethidium bromide were loaded into the gel and run in 1 X TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) at 50-60 V for 30-60 minutes. DNA was observed to be of high molecular weight when a single band greater than approximately 50 kilobases (kb) was seen under UV transillumination near the top of the gel; degraded or partially degraded DNA will appear as a long smear of fragments of varying sizes (Kirby, 1990).

Restriction digestion and electrophoresis

Genomic DNA samples (10 ug) were digested overnight at 37 °C with Pal I enzyme (Stratagene, La Jolla, CA) under conditions recommended by the manufacturer. Forty units (U) of enzyme were

added to DNA samples less than 95 ul in volume; 60 U were added to samples greater than 95 ul in volume. Pal I is an isoschizomer of Hae III which recognizes and cleaves the DNA sequence $^{5'}$ GG/CC $^{3'}$. Digestion was aided by the addition of 2 ul 0.1 M spermidine if the DNA sample was less than 95 ul in volume and 5 ul 0.1 M spermidine if the sample was greater than 95 ul in volume. Digested samples in volumes greater than 40 ul (or greater than 30 ul if a 36-well comb was used) were precipitated with Qwik-precip (Advanced Genetic Technologies Corp., Gaithersburg, MD) as recommended, recovered by centrifugation, washed with 70% ethanol, dried, and resuspended in 40 ul (or 30 ul) 1 X TE at 37 °C for at least 1 hour. Completion of digestion was verified on a 1% agarose gel by looking for a smear of DNA and not a single band of high molecular weight (greater than 50 kb) still near the well.

Digested samples in 40 ul (or 30 ul) 1 X TE plus 5 ul loading dye were loaded into 20 x 25 cm or 20 x 22 cm 0.9% agarose fingerprint gels and electrophoresed in pump-recirculated 1 X TAE buffer. A 250 g gel was prepared when using a 24-slot-6mm-wide comb and a 350 g gel was prepared when using a 36-slot-4mm-wide comb. Five ug of BstE II-digested lambda DNA (New England Biolabs, Beverly, MA) plus 10 ul of distilled water and 5 ul of loading dye (containing 0.2 ug/ul ethidium bromide) were loaded onto each gel as a molecular weight marker and visualized by UV transillumination. Gels were electrophoresed at 15-25 mA (10-20 V) for approximately 48 to 72 hours until the 3.7 kb marker fragment was between 14-15 cm from the wells and the 1.9 kb marker fragment was near the end of the gel. If the molecular weight marker could no longer be seen, 40 ul of ethidium bromide (10 mg/ml stock) were added prior to photographing the gel.

DNA was denatured in the gel by two 30 minute agitations in 500 ml of 0.5 M NaOH containing 0.15 M NaCl. This was followed with a neutralization by two 30 minute agitations in 500 ml of 0.5 M Tris containing 0.15 M NaCl (pH 7.7). Gels were placed on Whatman

3M paper, covered with plastic wrap, and dried under vacuum for 2 hours at 60 °C. Dried gels were placed in distilled water to remove the paper, wrapped in plastic wrap, and stored at -20°C until hybridized.

Hybridization and autoradiography

The synthetic oligonucleotides $(CAC)_5$ (Schafer, 1988) and $(GGCAGG)_4$ were used as probes for hybridization of fingerprint gels. The oligonucleotide $(GGCAGG)_4$ is based on the unstable mouse minisatellite sequence of GGCAGG (Mitani et. al., 1990). These probes detected high levels of polymorphism in *R. marmoratus* (Turner et. al., 1991, 1992b). Oligonucleotide probes were end-labeled using 20 units T4 polynucleotide kinase (New England Biolabs, Beverly, MA) at 37 °C for at least 1 hour with 50 uCi [gamma-^{32}P]ATP at 6000 Ci/mmol (Dupont New England Nuclear, Boston, MA). Hybridization buffer containing 5 X SSPE (0.15 M NaCl, 10 mM sodium phosphate monobasic, 1 mM EDTA), 0.1% SDS, and 1 ug sonicated transfer RNA was prepared. Twenty-five ml of hybridization buffer and 200 ng of labeled probe were added to a hybridization cylinder containing the rolled-up gel. In-gel hybridization was done overnight in a rotisserie at 45-47 °C for the oligonucleotide $(CAC)_5$ and at 53-55 °C for $(GGCAGG)_4$.

The first "stringency wash" consisted of agitation in 500 ml of 2 X SSPE containing 0.05% SDS at room temperature for 1 hour; the second "stringency wash" consisted of agitation in 500 ml of 5 X SSPE containing 0.1% SDS for 30-40 minutes at hybridization temperature.

Gels were blotted dry, wrapped in plastic wrap, and placed in a film cassette with intensifying screens and a piece of x-ray film (Kodak X-Omat AR) at -80 °C for 1-21 days depending on the level of activity. The gels were then stripped following the same denaturation and neutralization procedure as previously described (but in 1000 ml for 1 hour each) and stored wrapped at -20 °C.

FINGERPRINT ANALYSES

Design

An individual, heterozygous at some loci, will presumably result from a sexual mating unless the male and hermaphrodite parents belong to the same clonal line. However, most individuals in Florida populations belong to different clonal lines (Turner et. al., 1992b). These loci, also known as variable number of tandem repeat (VNTR) loci (Nakamura et. al., 1987), segregate in Mendelian fashion from parent to offspring as stable genetic markers (Jeffreys et. al., 1985; Burke and Bruford, 1987; Wetton et. al., 1987; Jeffreys and Morton, 1987; Kuhnlein et. al., 1990). To determine whether or not males contribute to sexual reproduction, progeny of laboratory-reared individuals from Florida and Brazil and progeny of field-caught individuals from the Belize Cays were surveyed for non-segregation or segregation of DNA fingerprint markers (VNTR loci) as indications of homozygosity or heterozygosity of the parent (Figure 4).

Along with a molecular weight marker, the same 2 individuals were run on every gel as standards (for digestion and hybridization). In a few instances, only 1 standard lane could be accomodated. As a validation of replicability of this technique, a fingerprint gel containing two separate DNA preps and 2 separate digestions (each with enough DNA for 2 lanes) from 3 separate fish was prepared (Figure 5).

Laboratory reference lines (parents and offspring) were run in adjacent lanes on a single gel with 1 or more lines per gel. From the 11 Florida lines and 1 Brazil line, a total of 156 individuals (siblings, parents, and offspring) were scored. Eight of the 12 lines were scored with $(CAC)_5$ and ten of the twelve lines were scored with $(GGCAGG)_4$. Six lines were scored with both probes.

Belize Cay lines (offspring only or parent and offspring) were run in adjacent lanes together on a single gel generally with 3 lines per gel. Five or six offspring (F_1) from each of the 30 field-caught

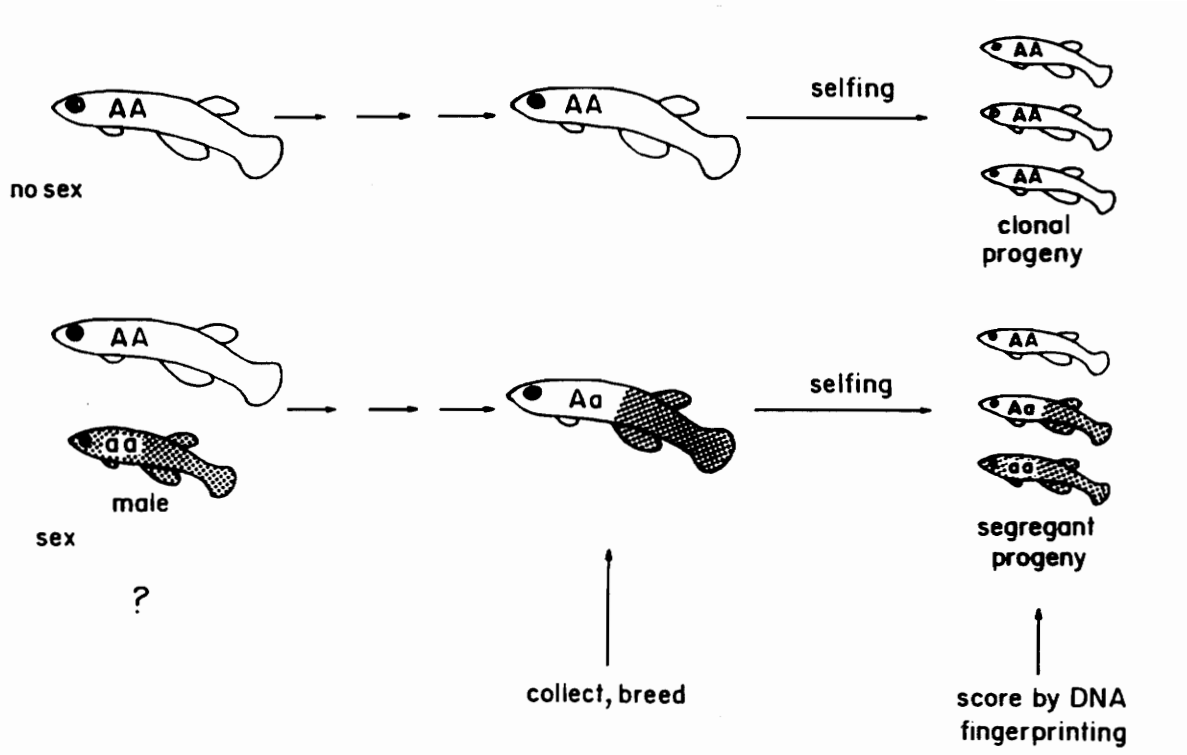


Figure 4. Experimental design for the study of the role of males in natural populations of *Rivulus marmoratus* . If there is no sexual reproduction in the population (top), the field-caught hermaphrodites will be homozygous (AA) as well as the resultant progeny. If males are contributing to sexual reproduction in the population by fertilizing infertile eggs emitted by hermaphrodites (bottom), some of the field-caught hermaphrodites will be heterozygous (Aa). When a heterozygous individual undergoes internal self-fertilization, the resultant progeny will have segregating genetic markers (AA, Aa, aa) which will be detected by DNA fingerprinting.

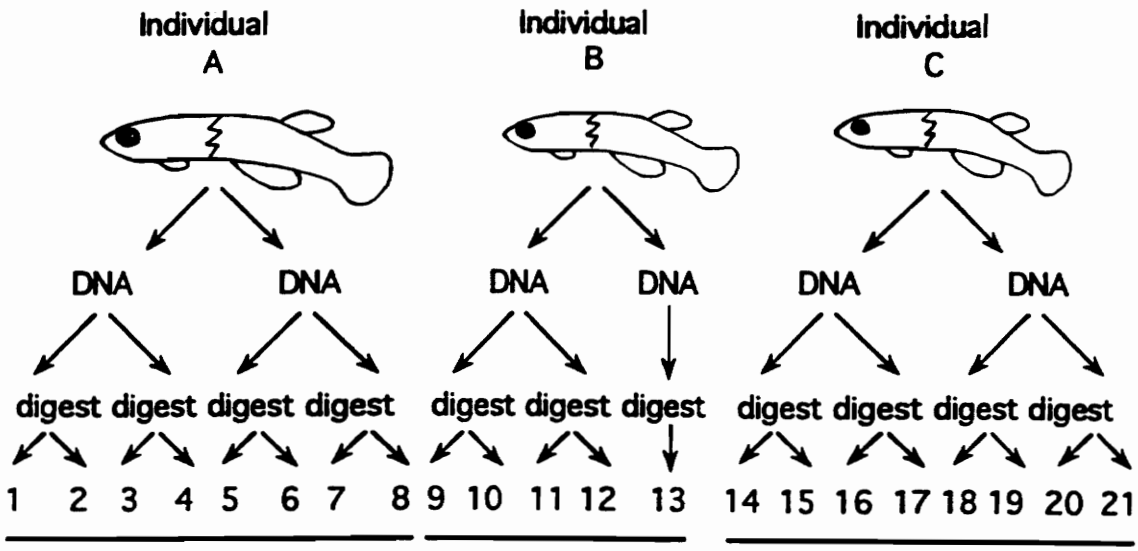


Figure 5. Diagram illustrating the experimental design for validation of the replicability of the DNA fingerprinting technique. DNA was isolated separately from both the head region and tail region of a single fish. Two DNA digestions were set up from each of the 2 DNA preps. Each digestion contained enough DNA for 2 lanes on the fingerprint gel. This was done using 3 different fish. Enough DNA for only 1 lane on the fingerprint gel was obtained from the back half of individual B because it was a small individual. All three individuals are Belize Cay fish. Individual A is an adult hermaphrodite collected on Twin Cays, individuals B (male) and C (hermaphrodite) are both offspring of a hermaphrodite collected on Coco Plum Cay.

hermaphrodites were analyzed as well as 13 of the parents (P) for a total of 180 individuals. Twenty of the 30 lines were scored using $(CAC)_5$ and 19 of the 30 lines were scored with $(GGCAGG)_4$. Nine lines were scored with both probes. A minimum of 5 offspring per Belize Cay line should reliably detect segregation of fingerprint loci. For any heterozygous locus in the parent where both fragments are resolved on the gel, the probability of observing the heterozygous genotype (both fragments detectable at the locus) per offspring is 0.5. It is unlikely that all 5 or 6 offspring will receive both fragments. The probability of all offspring receiving both fragments (that is, not detecting segregation in the offspring when it exists) is 0.031 (5 offspring) and 0.016 (6 offspring) (binomial probabilities). Therefore, if fingerprint loci in the parent are heterozygous, segregation of loci among the offspring will be detected.

The laboratory reference lines were included as controls in this study since males are very rare in natural populations in Florida (Davis et. al., 1990; Turner et. al., 1992a) and should contribute little to population variation. Any variation seen among the offspring would be due to mutational mechanisms rather than segregation of VNTR loci. Most mutations are probably germline in orgin (Jeffreys et. al., 1988), but somatic mutations can also occur (Thein et. al., 1987; Boltz et. al., 1990). Mutations result in the gain or loss of repeat units due to such mechanisms as unequal sister chromatid exchange or strand slippage during replication, but probably not unequal crossing over between homologous chromosomes (Wolff et. al., 1989, 1991). Parents were also included as a control against these mutational mechanisms causing fingerprint variation among the progeny. Any and all bands segregating among the progeny should be present in the parent. Any novel fragments found among the offspring but not in the parent as well as fragments found in the parent but not in any offspring were scored as mutations or artifacts and not included in analyses.

Fragment scoring and data analyses

All autoradiograms from hybridized fingerprint gels were scored by eye. Only clearly scorable bands (approximately 75-85% of the hybridized fragments) present or absent at a locus were counted.

For each line, two values were calculated: PSB, the proportion of bands in the parent segregating among the offspring (# segregating bands / # segregating bands + # fixed bands), and APD (Yuhki and O'Brien, 1990; Gilbert et. al., 1990), the average percent difference in band sharing among the offspring. The APD of each line was calculated using all pairwise comparisons between the offspring. The APD is a rough estimate of heterozygosity (Gilbert et. al., 1991) and is the additive inverse of the average percent similarity (Wetton et. al., 1987; Lynch, 1988 and 1990). The average percent difference, as opposed to the average percent similarity, was chosen as a value for comparison because it will vary directly (and not inversely) with increasing levels of heterozygosity.

To calculate APD:

$$APD = \frac{1}{C} \sum_{i=1}^{C} PD_i$$

where C = the number of pairwise comparisons, and PD is the percent difference between any two individuals. To calculate PD:

$$PD = \left[\frac{N_{ab}}{N_a + N_b} \right] X\ 100$$

where Nab = the number of fingerprint bands that differ between individuals a and b and N_a and N_b = the total number of scorable bands in individuals a and b respectively.

If males have no role in the breeding system, there will be no variation in the fingerprint banding patterns among the offspring of any given hermaphrodite. The estimated proportion of bands in the parent segregating among the offspring will be 0. There will be no differences in band sharing among the offspring estimated by the average percent difference. Lines with PSB and APD values of 0 were scored as homozygous.

However, if males are involved in the breeding system, there will be Mendelian segregation of fingerprint bands among the offspring. The estimated proportion of bands in the parent segregating among the offspring will be greater than zero. There will be band differences among the offspring estimated by the average percent difference. Lines with PSB and APD values greater than zero were scored as heterozygous.

RESULTS

DNA FINGERPRINTING

Replicability

No variation was evident in the fingerprint patterns of the same fish (standards) run on different gels (except in hybridization efficiencies). From the replicability experiment, no variation in fingerprint patterns was evident of the same fish on the same gel regardless of tissue from which the DNA was extracted and regardless of digestion (Figure 6).

Artifacts

The frequencies of artifact bands, bands in offspring not in parents and bands in parents not in offspring, detected on fingerprint gels differed between the 2 probes (Table 2). No new bands were detected among Florida and Brazil laboratory lines using $(CAC)_5$, while 12 new offspring bands and 5 new parental bands were detected with $(GGCAGG)_4$. If these bands are novel mutations and not artifacts (such as intensity differences), then the mutation rate with $(GGCAGG)_4$ in Florida and Brazil lines is approximately 6.7×10^{-3} for novel offspring bands and 1.0×10^{-2} for novel parental bands (Table 2). All 12 new offspring bands occurred within the same line and represented the same size fragment in each of 12 offspring from 4 different parents. Four of the five new parental bands also occurred within this line and represented the same size fragment in each parent (but different from the novel fragment in the offspring). Neither somatic nor germline mutations in the parents, however, could account for this observation. The other new parental band probably represented a new length allele (resulting from a somatic mutation).

No new parental bands were detected in the Belize Cay lines while two new offspring bands were detected with $(CAC)_5$

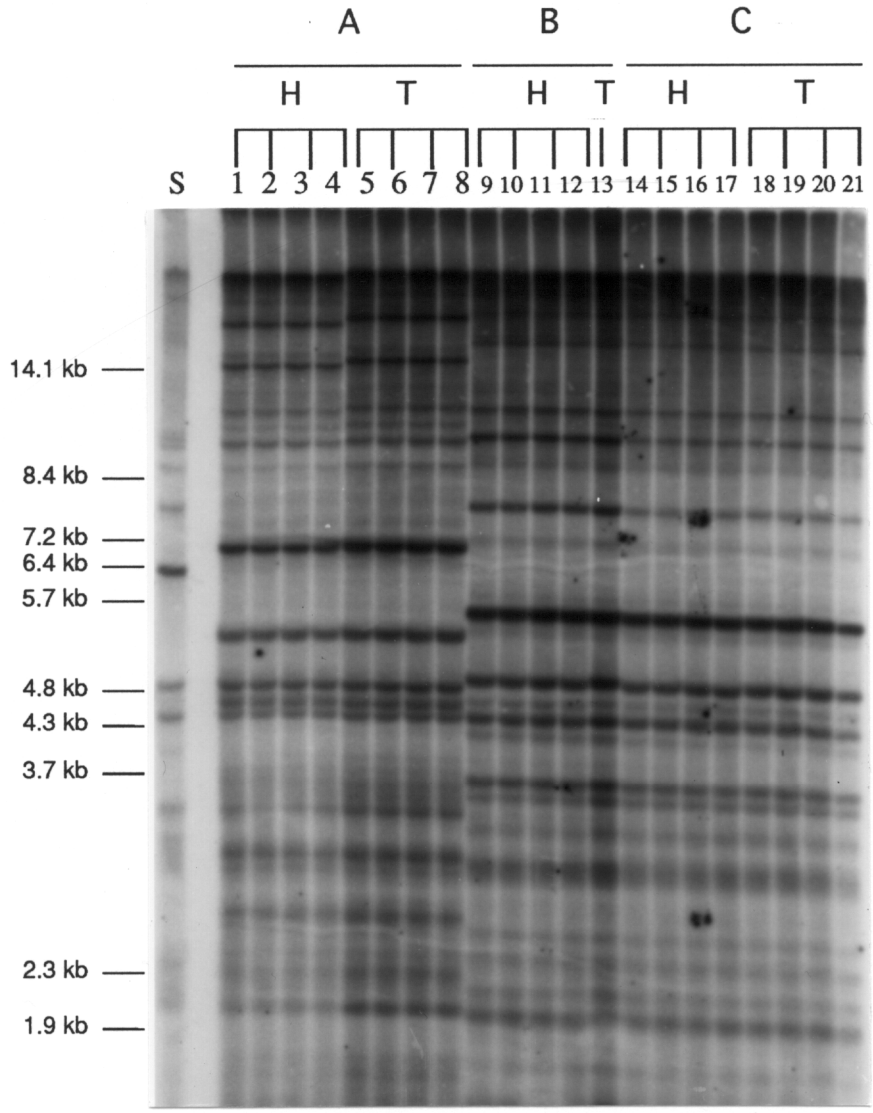


Figure 6. Autoradiogram of genomic DNA from 3 Belize Cay fish demonstrating the replicability of the DNA fingerprinting technique [(GGCAGG)4 probe]. Lanes 1-8 contain DNA from Individual A, lanes 9-13 contain DNA from individual B, and lanes 14-21 contain DNA from individual C. H = DNA isolated from the head region; T = DNA isolated from the tail region. Refer again to Fig. 5 for detailed diagram of design.

Table 2. Frequencies of artifact bands (# novel bands/total # bands). Fingerprint gels included DNA from both parent and offspring on the same gel. (See Appendix A for detailed data on each line analyzed.)

Bands in offspring not found in parent

Florida and Brazil		
$(CAC)_5$	0/1199 =	$< 8.3 \times 10^{-4}$
$(GGCAGG)_4$	12/1789 =	6.7×10^{-3}
Belize Cays		
$(CAC)_5$	2/663 =	3.0×10^{-3}
$(GGCAGG)_4$	6/1057 =	5.7×10^{-3}

Bands in parent not found in offspring

Florida and Brazil		
$(CAC)_5$	0/288 =	$< 3.5 \times 10^{-3}$
$(GGCAGG)_4$	5/496 =	1.0×10^{-2}
Belize Cays		
$(CAC)_5$	0/139 =	$< 7.2 \times 10^{-3}$
$(GGCAGG)_4$	0/203 =	$< 4.9 \times 10^{-3}$

(3.0×10^{-3}), and 6 new bands were detected using $(GGCAGG)_4$ (5.7×10^{-3}) (Table 2). Four of the 6 new offspring bands were within 1 line and represented the same size fragment in 4 of 6 offspring, possibly due to a germline mutation in the parent. All of the Belize Cay novel fragments probably represent new length alleles.

Probe correlation

Fingerprint gels were hybridized with the oligonucleotide probes $(CAC)_5$ and/or $(GGCAGG)_4$. Scored fragments ranged from approximately 1.9 kb to 25 kb in size. The probe $(CAC)_5$ detected an average of 20.8 ± 0.3 (s.e.m.) bands while $(GGCAGG)_4$ detected an average of 22.9 ± 0.3 bands (Table 3). No line was scored as homozygous with one probe and heterozygous with the other, although the banding patterns of the same individuals differed with the 2 probes (Figure 7).

A correlation analysis was performed using 8 heterozygous Belize Cay lines scored with both probes to determine whether the PSB and APD values were a function of the probe used or whether each was an accurate estimate of heterozygosity (Figure 8). There was a significant correlation among the proportion of segregating bands with both probes ($p=0.02$, Pearson's coefficient $r=0.80$; $p=0.002$, Spearman's $rho=0.91$). There was also a significant correlation among the average percent difference values with both probes (Pearson's coefficient $p=0.006$, $r=0.86$; $p=0.006$, Spearman's $rho = 0.86$). The R^2 values were fairly high ($R^2=0.74$, $R^2=0.64$) which indicate that the heterozygosity measure can be estimated with either probe. Since the correlation was not perfect, the PSB and APD values were averaged when lines were scored with both probes. PSB and APD values will be presented from the 2 probes separately, and by combining probe data so comparisons could be made using all 12 Florida and Brazil lines and all 30 Belize Cay lines.

Table 3. Means, standard errors, and ranges of scorable fingerprint bands per individual fish. The Florida and Brazil lines were combined. Sample size indicates the total number of fish whose fingerprint pattern was analyzed.

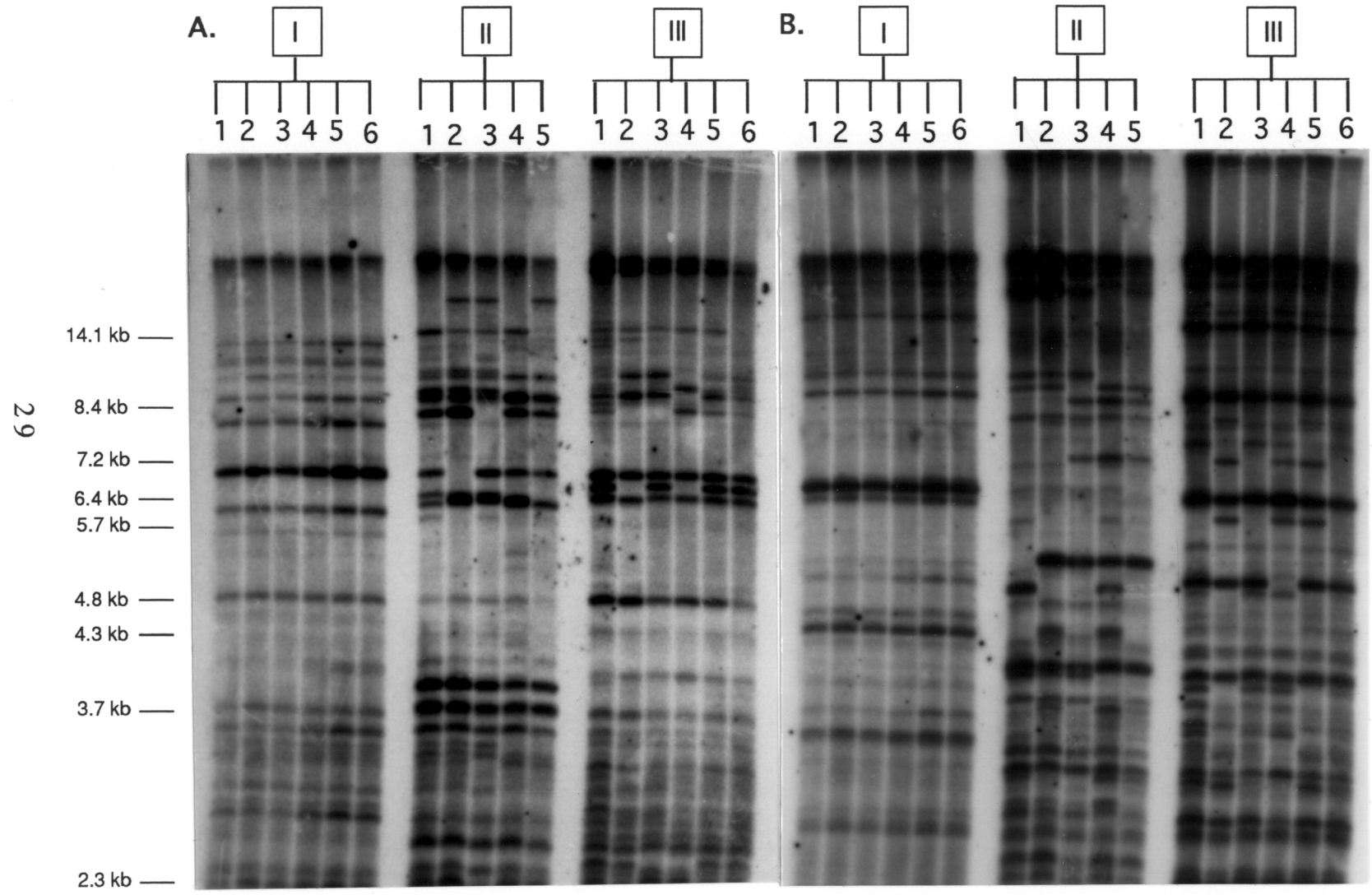
(CAC)5

	sample size	mean (s.e.)	range
total	n = 224	20.8 (0.3)	12-32
Florida and Brazil	n = 105	18.7 (0.3)	15-25
Belize Cays	n = 119	22.6 (0.4)	12-32

(GGCAGG)4

	sample size	mean (s.e.)	range
total	n = 240	22.9 (0.3)	11-33
Florida and Brazil	n =126	23.4 (0.3)	18-32
Belize Cays	n = 114	22.3 (0.6)	11-32

Figure 7. Autoradiogram of genomic DNA of offspring from 3 Belize Cay hermaphrodites illustrating DNA fingerprint patterns. A. $(CAC)_5$ probe, B. $(GGCAGG)_4$ probe. The offspring of individual I have identical fingerprint markers while the offspring of individuals II and III have segregating fingerprint markers. Individual I was scored as homozygous with both probes; individuals II and III were scored as heterozygous with both probes. Individual I was collected on Pelican Cay and individuals II and III were collected on Twin Cays.


A.
I
II
III
1 2 3 4 5 6
1 2 3 4 5
1 2 3 4 5 6
B.
I
II
III
1 2 3 4 5 6
1 2 3 4 5
1 2 3 4 5 6
14.1 kb
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6.4 kb
5.7 kb
4.8 kb
4.3 kb
3.7 kb
2.3 kb

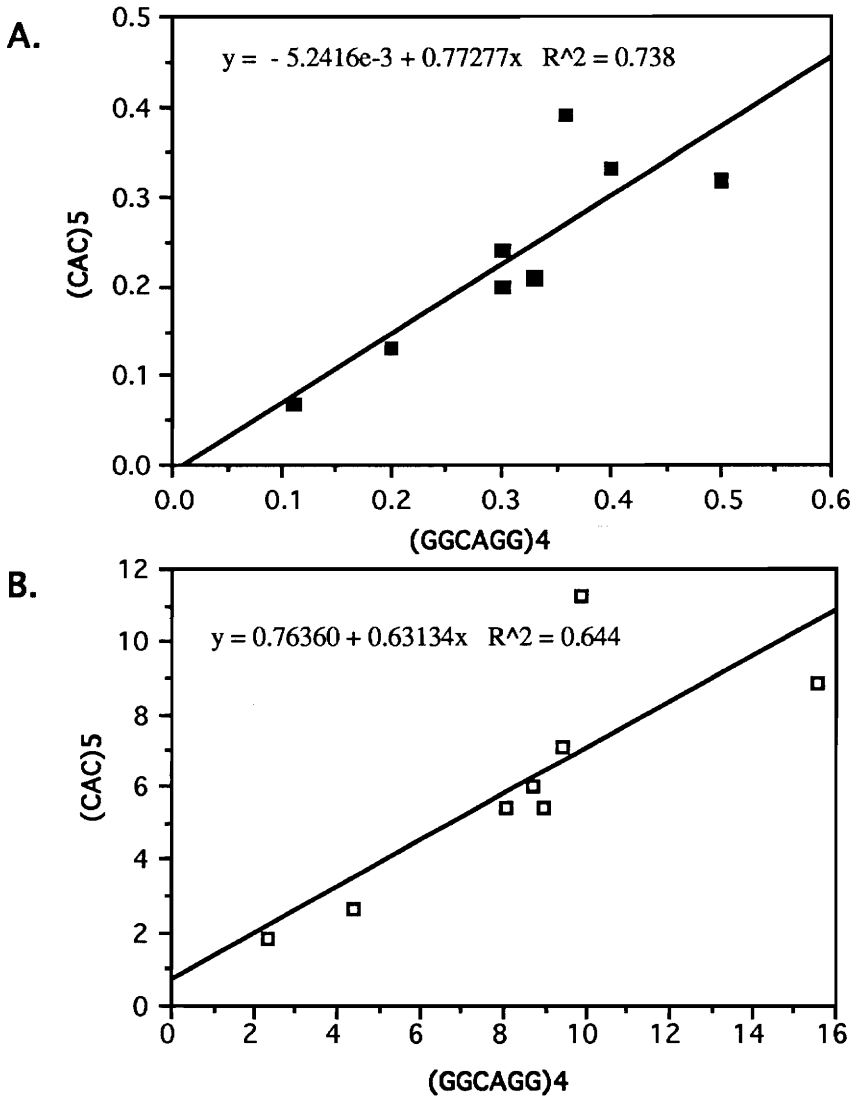

Figure 8. Scatterplots of PSB and APD values from 8 heterozygous Belize Cay lines scored with the oligonucleotide probes (CAC)5 and (GGCAGG)4. Sample size = 8. A.) Proportion of segregating bands (PSB). B.) Average percent difference (APD) among the offspring.

Fingerprint patterns

There was no variation among the offspring in DNA fingerprint patterns in any of the 12 Florida and Brazil lines. The fingerprint patterns of all individuals surveyed within a line were identical as seen in Figures 9 & 10. The DNA fingerprint patterns of Belize Cay lines were different from the DNA fingerprint patterns of Florida and Brazil lines. There was variation among the offspring in DNA fingerprint patterns in 25 of the 30 Belize Cay lines (83%) (Figures 11 & 12).

Proportion of segregating bands

The proportion of bands in the parent segregating among the offspring was determined for each Florida and Brazil line and each Belize Cay line using the data from the 2 probes separately and also combining the data (Table 4). Both methods gave similar results. Looking at the results with either probe (Figure 13) and both probes combined (Figure 14), the PSB data using either method indicated fingerprint bands were not segregating among the offspring in any of the 12 Florida and Brazil lines while 25 of the 30 Belize Cay lines had fingerprint markers that were segregating among the offspring. In some Belize Cay lines, up to half of the bands in the offspring were segregating. The mean proportion of segregating bands among the offspring in Florida and Brazil lines was 0.0 which contrasts the mean PSB in Belize Cay lines of 0.25 ± 0.03 (Table 4). The average proportion of segregating bands in Belize Cay lines was significantly greater than zero [$(CAC)_5$: $p<0.001$, $T=8.21$; $(GGCAGG)_4$: $p<0.001$, $T=6.49$, combined: $p<0.0001$, $T=9.94$].

The PSB values were also compared between the Belize Cay lines using the data from the 2 probes separately and also combining the data (Table 5). Lines from Twin Cays had the highest values. Lines from two of the islands - Pelican Cay and Coco Plum Cay - were similar to the Florida and Brazil lines without any bands segregating

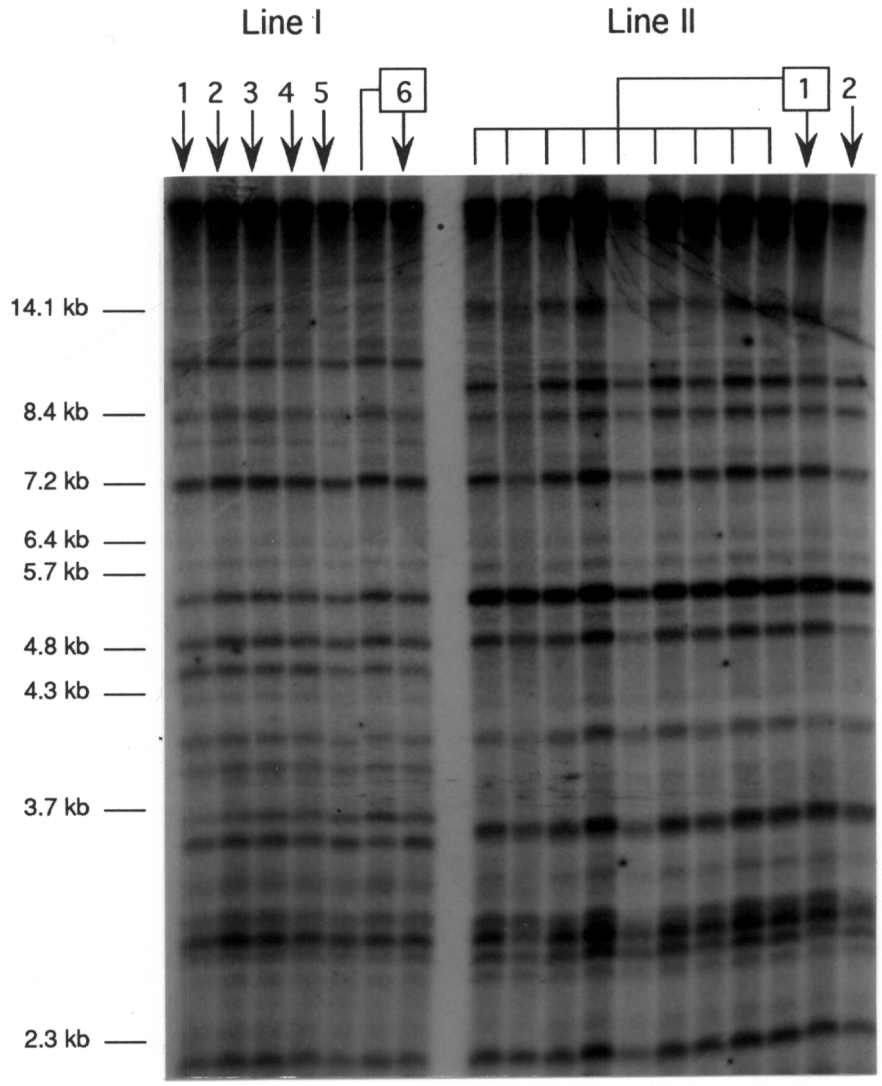

Figure 9. Autoradiogram of genomic DNA of fish from laboratory-maintained Florida lines demonstrating the clonal inheritance and clonal stability of DNA fingerprint markers [(CAC)5 probe]. Line I consists of 6 siblings from a long-term line originating from Miami and 1 offspring of individual 6. Line II consists of 2 F_1 offspring from an individual collected in Everglades National Park and 9 subsequent offspring of individual 1.

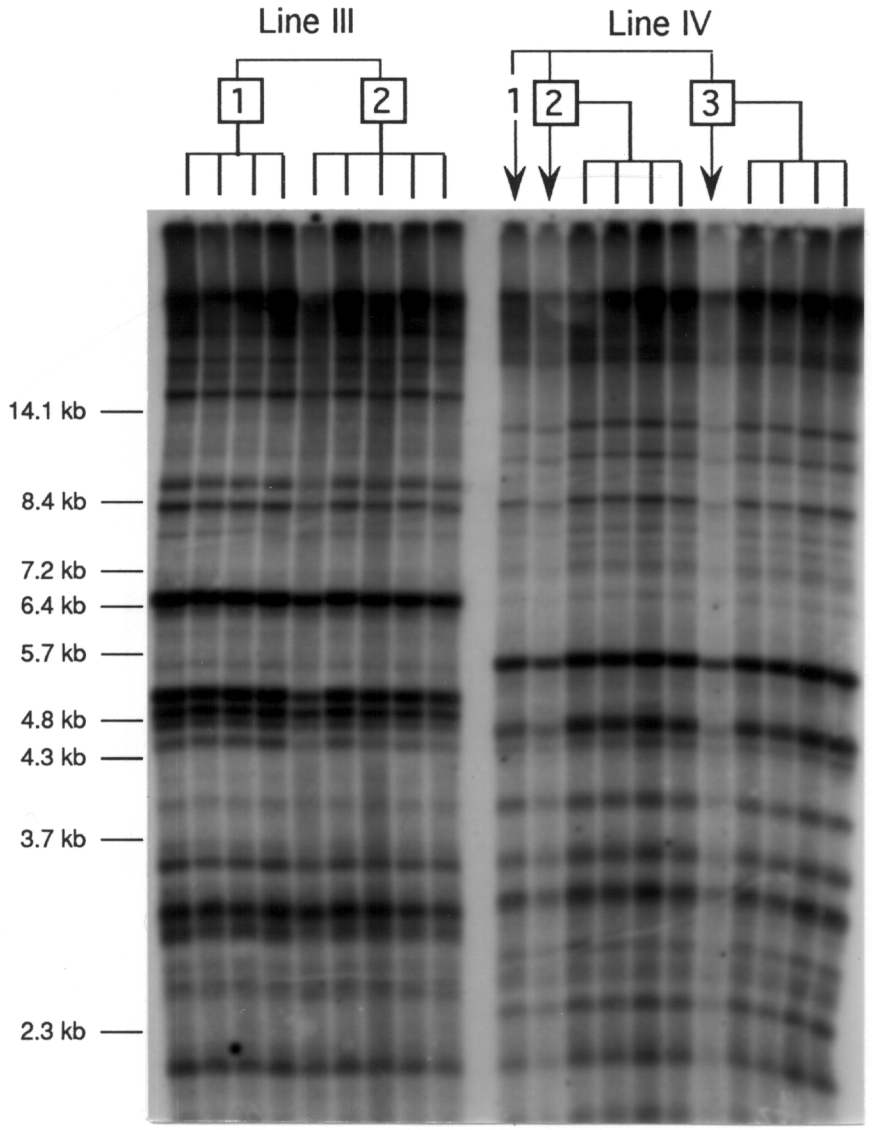

Figure 10. Autoradiogram of genomic DNA of fish from laboratory-maintained Florida lines demonstrating the clonal inheritance and clonal stability of fingerprint markers [$(GGCAGG)_4$ probe]. Line III consists of 9 offspring from 2 separate individuals. Individuals 1 and 2 are siblings from a long-term line originating from Marco Is./ Rookery Bay. Line IV consists of 3 F_1 offspring from an individual collected on No Name Key and 4 subsequent offspring each of individuals 2 and 3.

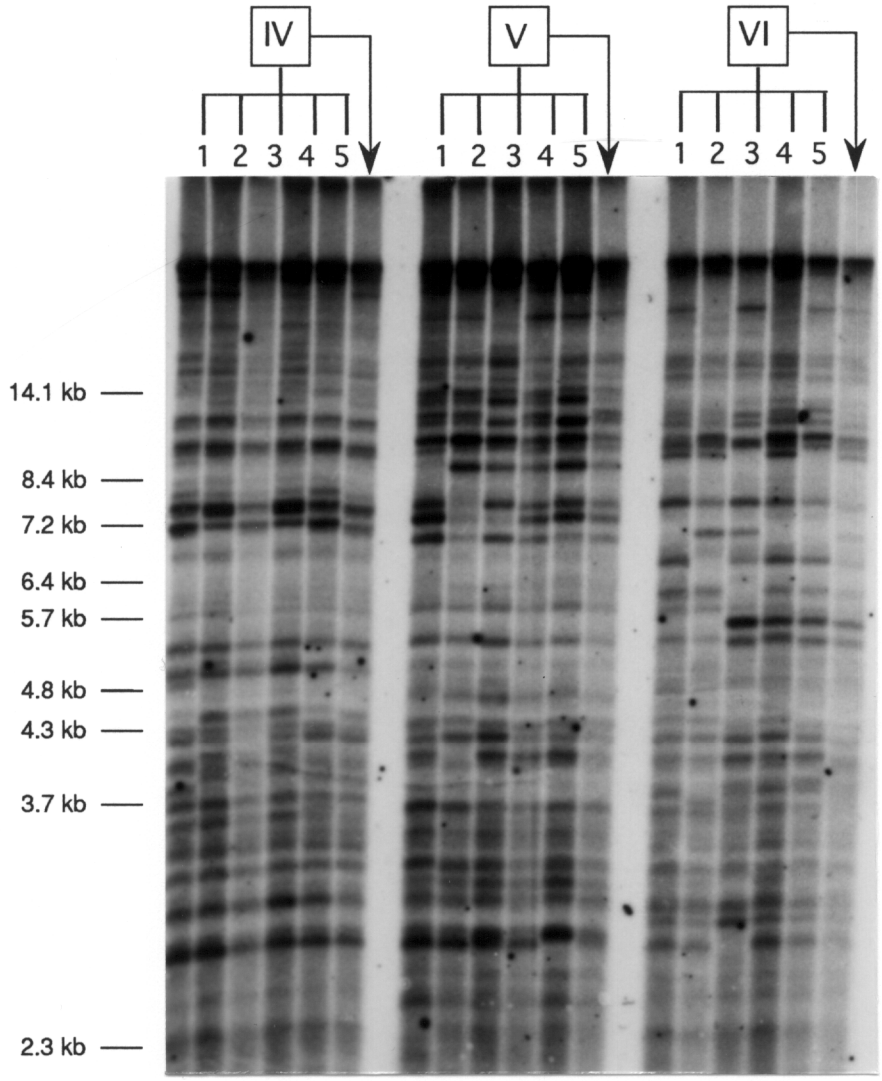

Figure 11. Autoradiogram of genomic DNA of hermaphrodites from the Belize Cays and offspring demonstrating DNA fingerprint variation among the offspring [(CAC)5 probe]. DNA fingerprint markers are segregating among the 5 offspring of all 3 parents (IV, V, VI) field-caught on Twin Cays.

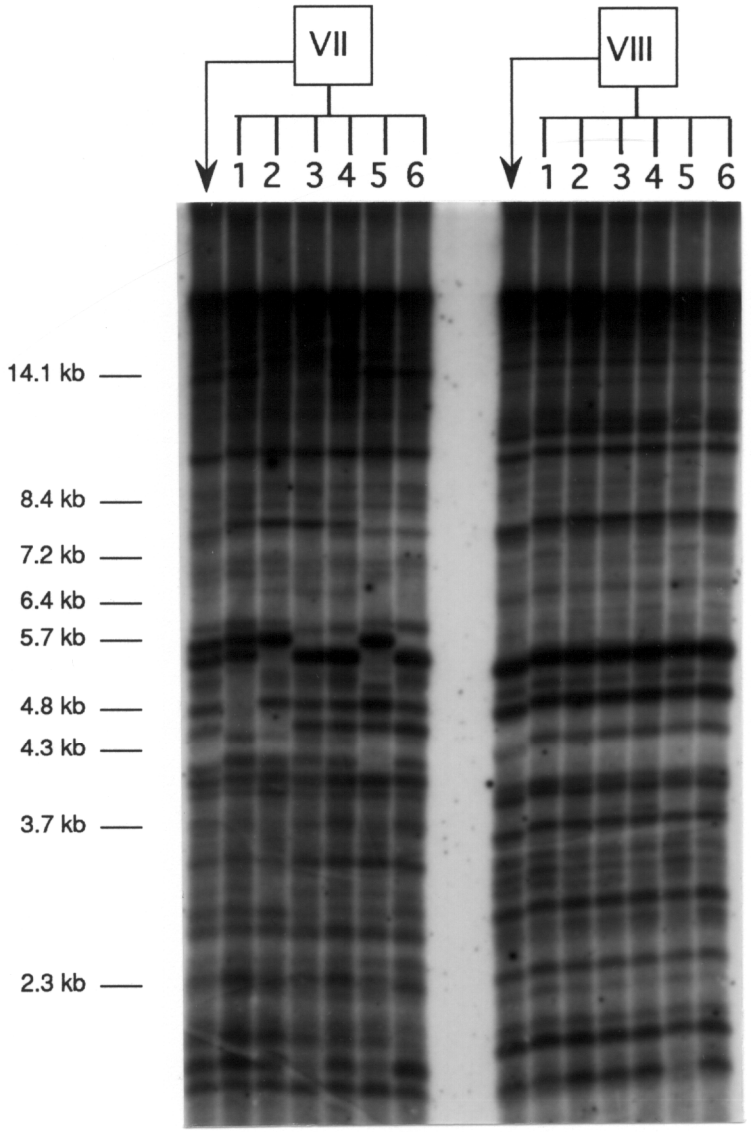

Figure 12. Autoradiogram of genomic DNA of hermaphrodites from the Belize Cays and offspring demonstrating segregation and non-segregation of DNA fingerprint markers among the offspring [$(GGCAGG)_4$ probe]. Fingerprint markers are segregating among the offspring of parent VII but are not segregating among the offspring of parent VIII. Individual VII was collected on Twin Cays; individual VIII was collected on Coco Plum Cay.

Table 4. Means, standard errors, and ranges of the proportion of segregating bands (PSB) among the offspring. PSB = (# bands segregating / # bands segregating + # bands fixed). Sample size indicates the number of lines (parent and laboratory-derived offspring or offspring only) analyzed.

	sample size	mean PSB (s.e.)	range
$(CAC)_5$			
Florida and Brazil	n= 8 lines	0.0 (--)	(--)
Belize Cays	n = 20 lines	0.25 (0.03)	0.0-0.44
$(GGCAGG)_4$			
Florida and Brazil	n = 10 lines	0.0 (--)	(--)
Belize Cays	n = 19 lines	0.25 (0.04)	0.0-0.50
combined			
Florida and Brazil	n = 12 lines	0.0 (--)	(--)
Belize Cays	n = 30 lines	0.25 (0.03)	0.0-0.50

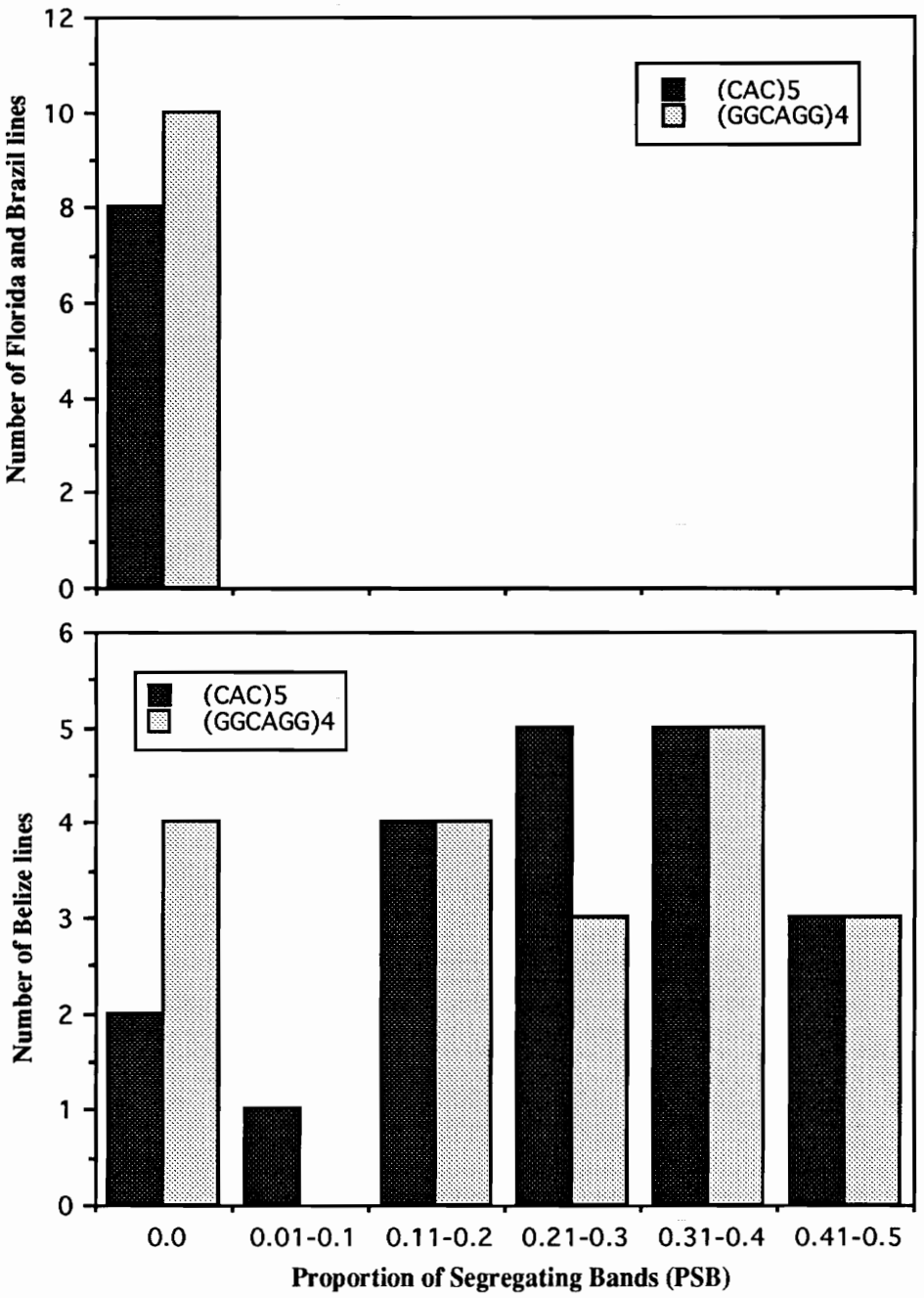

Figure 13. Distribution of the proportion of segregating bands (PSB) among the offspring of Florida and Brazil lines (combined) and Belize Cay lines estimated with either (CAC)5 or (GGCAGG)4. Florida and Brazil lines are shown at the top; Belize Cay lines are shown at the bottom.

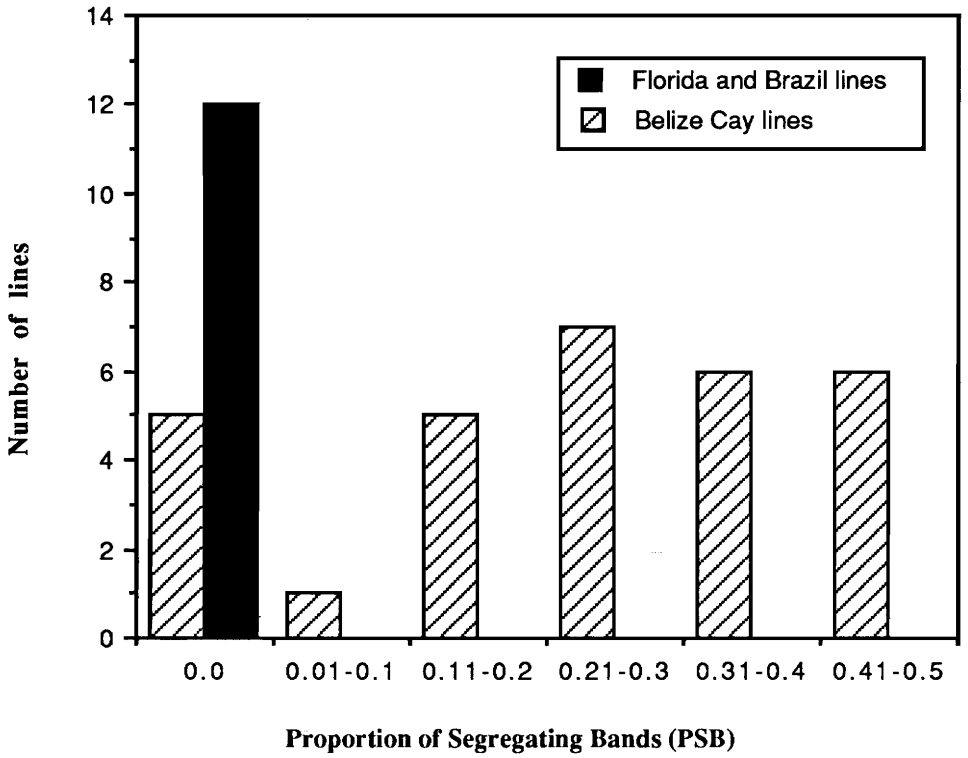

Figure 14. Distribution of the proportion of segregating bands (PSB) among the offspring of Florida and Brazil lines (combined) and Belize Cay lines estimated with $(CAC)_5$ and/or $(GGCAGG)_4$.

Table 5. Means, standard errors, and ranges of the proportion of segregating bands (PSB) among the offspring within Belize Cay lines.

	sample size	mean PSB (s.e.)	range
$(CAC)_5$			
Pelican Cay	n = 1 line	0.0 (--)	(--)
Ragged Cay	n = 1 line	0.43 (--)	(--)
Coco Plum Cay	n = 1 line	0.0 (--)	(--)
Twin Cays	n = 17 lines	0.27 (0.03)	0.07-0.44
$(GGCAGG)_4$			
Pelican Cay	n = 1 line	0.0 (--)	(--)
Ragged Cay			
Coco Plum Cay	n = 3 lines	0.0 (0.0)	(--)
Twin Cays	n = 15 lines	0.32 (0.03)	0.11-0.50
combined			
Pelican Cay	n = 1 line	0.0 (--)	(--)
Ragged Cay	n = 1 line	0.43 (--)	(--)
Coco Plum Cay	n = 4 lines	0.0 (0.0)	(--)
Twin Cays	n = 24 lines	0.30 (0.02)	0.09-0.50

among the offspring (PSB=0). All of the lines from Twin Cays had PSB values greater than zero. In Twin Cays lines, a minimum of 2 bands and a maximum of 18 bands were detected as segregating among the offspring. Using the combined probe data, Figure 15 illustrates that an island either contained all lines with PSB values equal to 0 or all lines with PSB values greater than 0. When comparing Belize Cay lines from Coco Plum Cay and Twin Cays using the combined scores, the 2 groups were significantly different ($p=0.002$, Mann Whitney U test statistic = 96). No other comparisons could be made between the islands due to small sample sizes.

Average percent differences in band sharing

The average percent difference (APD) among the offspring was calculated for each Florida and Brazil line and each Belize Cay line using the data from the 2 probes separately and also combining the data (Table 6). Both methods gave similar results. Results of APD scores were similar to the PSB scores. Looking at the results with either probe (Figure 16) and both probes combined (Figure 17), the APD data using either method indicated there were no differences in band sharing among the offspring in any of the 12 Florida and Brazil lines while there were differences in band sharing among the offspring in 25 of the 30 Belize Cay lines. In at least 1 Belize Cay line, the average percent difference among the offspring was 15% (which conversely indicates that the offspring were approximately 85% similar to one another). The combined mean APD value in Florida and Brazil lines was 0.0% which again differs from the mean APD in Belize Cay lines of $6.85\% \pm 0.82$ (Table 6). The mean APD in Belize Cay lines was significantly greater than zero [$(CAC)_5$: $p<0.0001$, $T=7.47$; $(GGCAGG)_4$: $p<0.0001$, $T=6.00$, combined: $p<0.0001$, $T=8.38$].

The APD values were also compared between Belize Cay lines using the data from the 2 probes separately and also combining the data (Table 7). Lines from Twin Cays had the highest values.

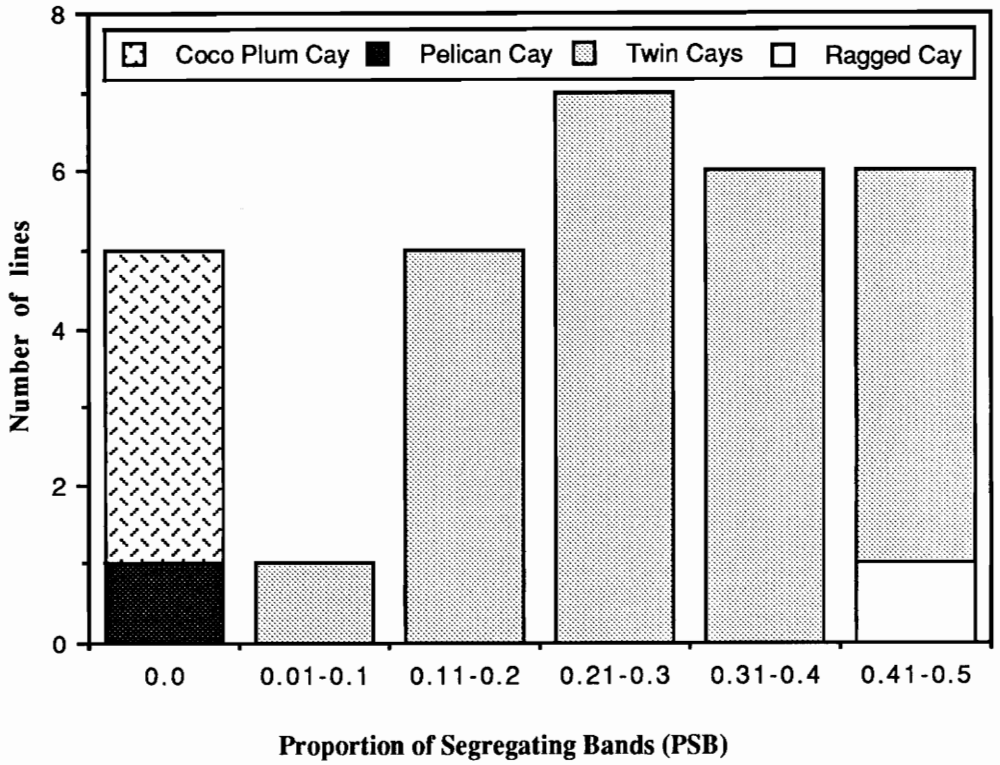

Figure 15. Distribution of the proportion of segregating bands (PSB) among the offspring of Belize Cay lines from Coco Plum Cay, Pelican Cay, Twin Cays, and Ragged Cay using the oligonucleotide probes $(CAC)_5$ and/or $(GGCAGG)_4$.

Table 6. Means, standard errors, and ranges of the average percent difference (APD) in fingerprint band sharing among the offspring. APD was calculated using all pairwise comparisons among the offspring. For all Belize Cay lines, 5 or 6 offspring from each hermaphrodite were analyzed.

	sample size	mean APD (s.e.)	range
(CAC)5			
Florida and Brazil	n = 8 lines	0.0 (--)	(--)
Belize Cays	n = 20 lines	6.67 (0.89)	0.0-13.50
(GGCAGG)4			
Florida and Brazil	n = 10 lines	0.0 (--)	(--)
Belize Cays	n = 19 lines	6.84 (1.14)	0.0-15.53
combined			
Florida and Brazil	n = 12 lines	0.0 (--)	(--)
Belize Cays	n = 30 lines	6.85 (0.82)	0.0-15.09

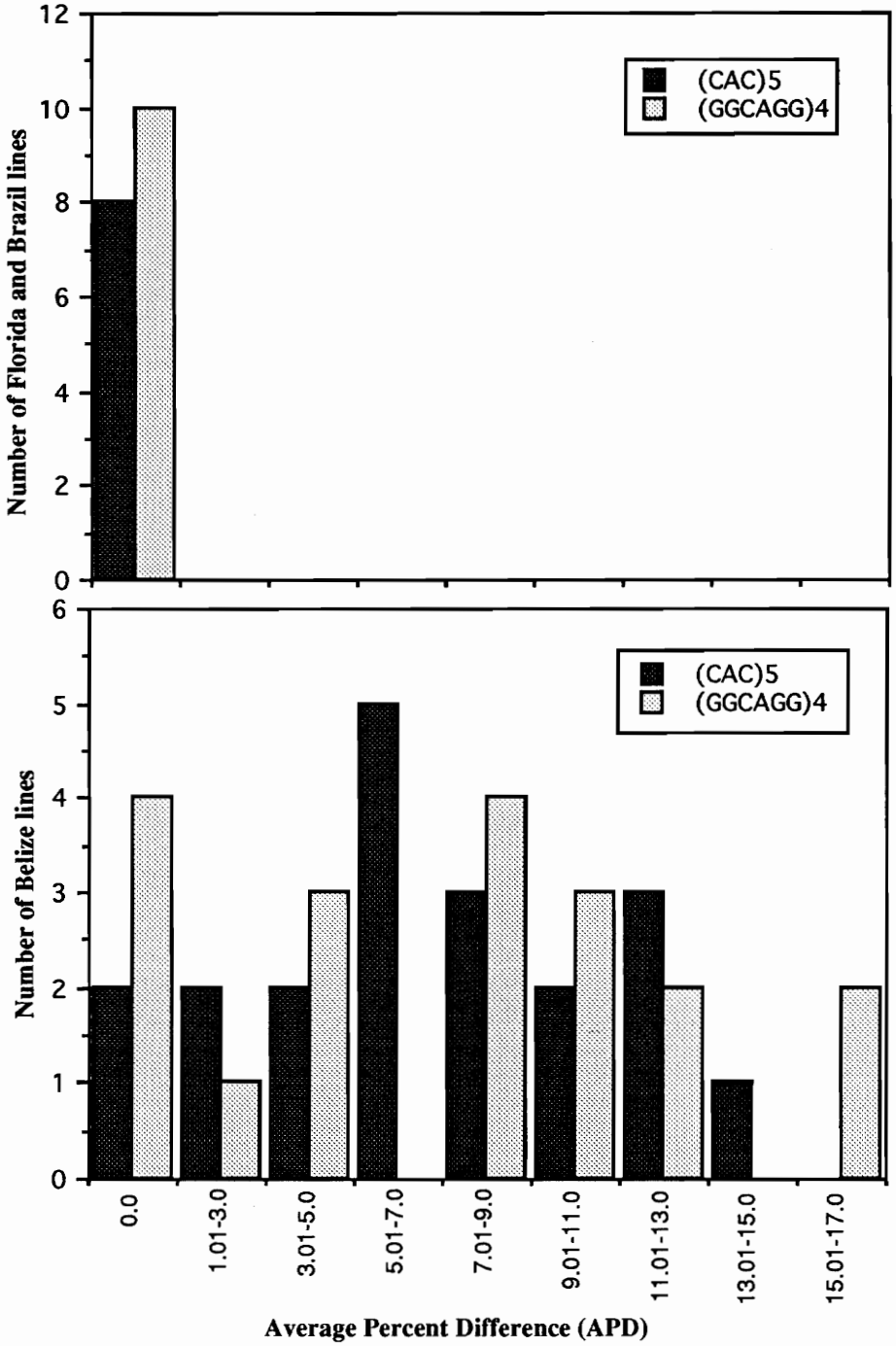

Figure 16. Distribution of the average percent difference (APD) values among the offspring of Florida and Brazil lines (combined) and Belize Cay lines estimated with either (CAC)5 or (GGCAGG)4.

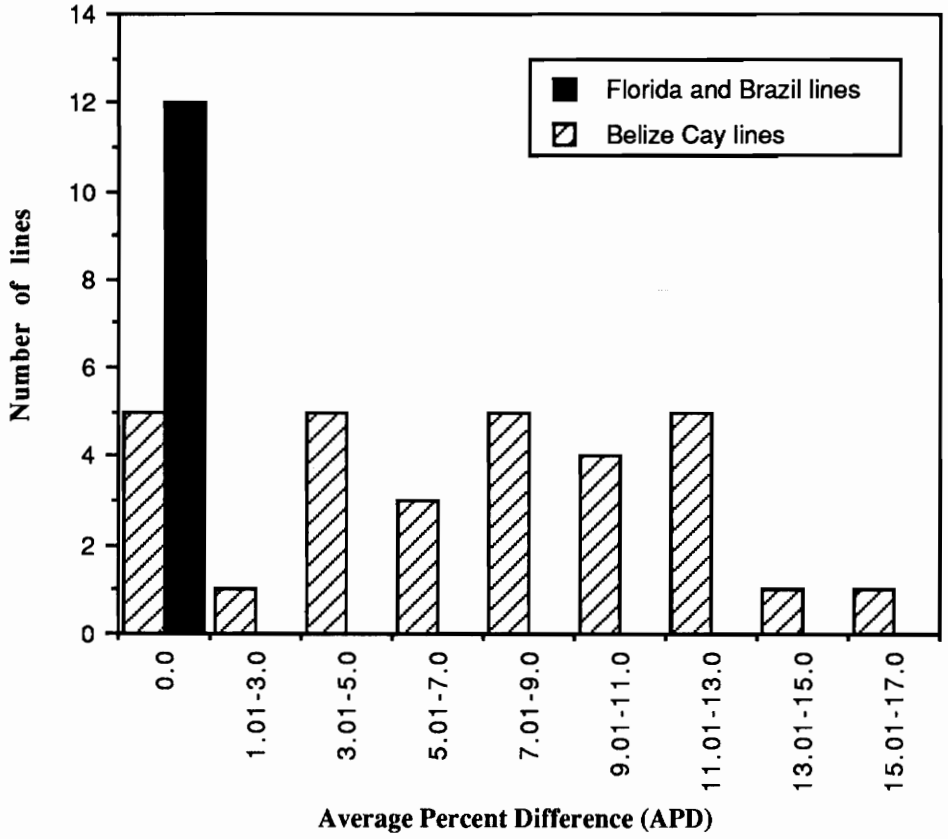

Figure 17. Distribution of the average percent difference (APD) values among the offspring of Florida and Brazil lines (combined) and Belize Cay lines estimated with $(CAC)_5$ and/or $(GGCAGG)_4$.

Table 7. Means, standard errors, and ranges of the average percent difference (APD) in band sharing among the offspring in Belize lines from the 4 islands.

	sample size	mean APD (s.e.)	range
$(CAC)_5$			
Pelican Cay	n = 1 line	0.0 (--)	(--)
Ragged Cay	n = 1 line	12.41 (--)	(--)
Coco Plum Cay	n = 1 line	0.0 (--)	(--)
Twin Cays	n = 17 lines	7.12 (0.81)	1.85-13.50
$(GGCAGG)_4$			
Pelican Cay	n = 1 line	0.0 (--)	(--)
Ragged Cay			
Coco Plum Cay	n = 3 lines	0.0 (0.0)	(--)
Twin Cays	n = 15 lines	8.67 (0.99)	2.30-15.53
combined			
Pelican Cay	n = 1 line	0.0 (--)	(--)
Ragged Cay	n = 1 line	12.41 (--)	(--)
Coco Plum Cay	n = 4 lines	0.0 (0.0)	(--)
Twin Cays	n = 24 lines	8.05 (0.71)	2.08-15.09

Lines from two of the islands - Pelican Cay and Coco Plum Cay - were similar to the Florida and Brazil lines without any band differences among the offspring (APD=0). All of the lines from Twin Cays had APD values greater than zero. Using the combined probe data, Figure 18 illustrates that an island either contained all lines with APD values equal to 0 or all lines with APD values greater than 0. When comparing Belize Cay lines from Coco Plum Cay and Twin Cays using the combined scores, the 2 groups were significantly different ($p=0.002$, Mann Whitney U test statistic = 96). No other statistical comparisons could be made between islands due to small sample sizes.

Detection of outcrossing

Since all lines from Florida and Brazil surveyed in this study had both PSB and APD values of 0, they were scored as homozygous. Five of the 30 Belize Cay lines had PSB and APD values of 0 and were scored as homozygous while the remaining 25 lines had PSB and APD values greater than zero and were scored as heterozygous. Even though not all of the Belize Cay lines were heterozygous (Figure 19), the proportion of heterozygous lines from the Belize Cays was significantly greater than the proportion of heterozygous lines from Florida and Brazil (combined) ($p<0.0001$, Fisher's Exact Test).

Looking more closely at the Belize Cay lines, there was also a difference between the 4 islands. Fish from a particular island were either all homozygous or all heterozygous. Homozygous fish were only found on the islands of Pelican Cay and Coco Plum Cay, while heterozygous fish were only found on the islands of Ragged Cay and Twin Cays (Figure 20). The proportion of heterozygous lines from Twin Cays was significantly greater than the proportion of heterozygous lines from Coco Plum Cay ($p<0.0001$, Fisher's Exact Test). Other statistical comparisons could not be made due to the small sample sizes.

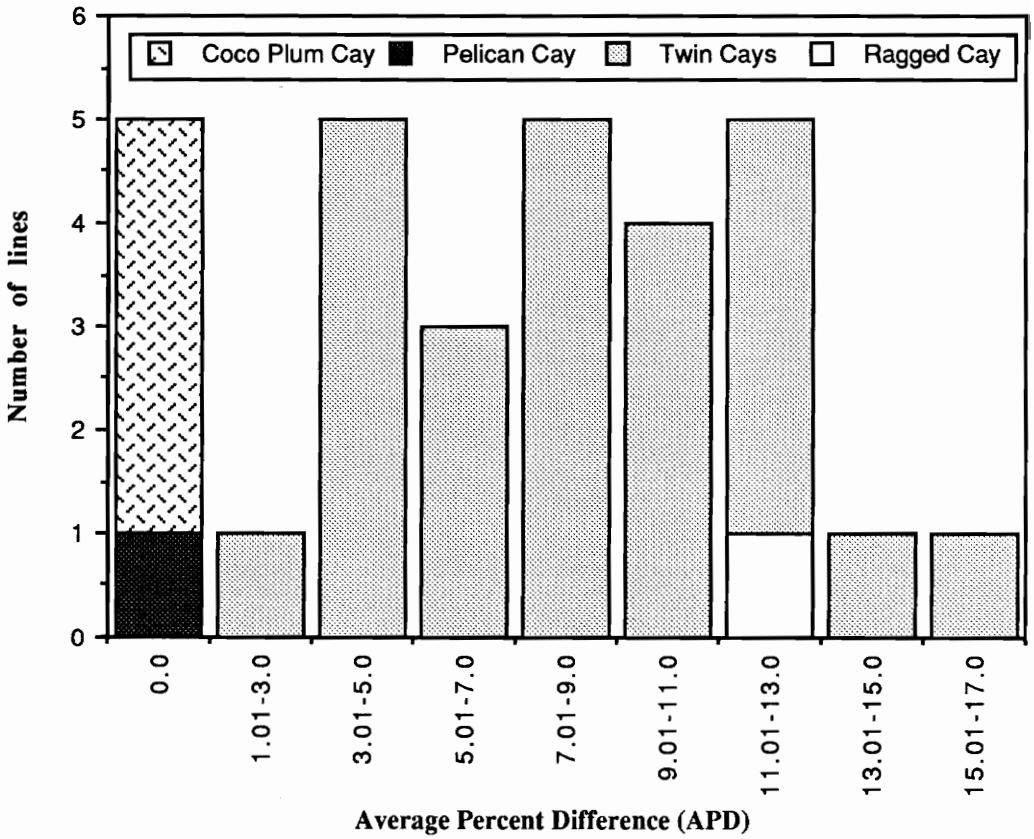

Figure 18. Distribution of the average percent difference (APD) values among the progeny of Belize Cay lines from Coco Plum Cay, Pelican Cay, Twin Cays, and Ragged Cay using the oligonucleotide probes $(CAC)_5$ and/or $(GGCAGG)_4$.

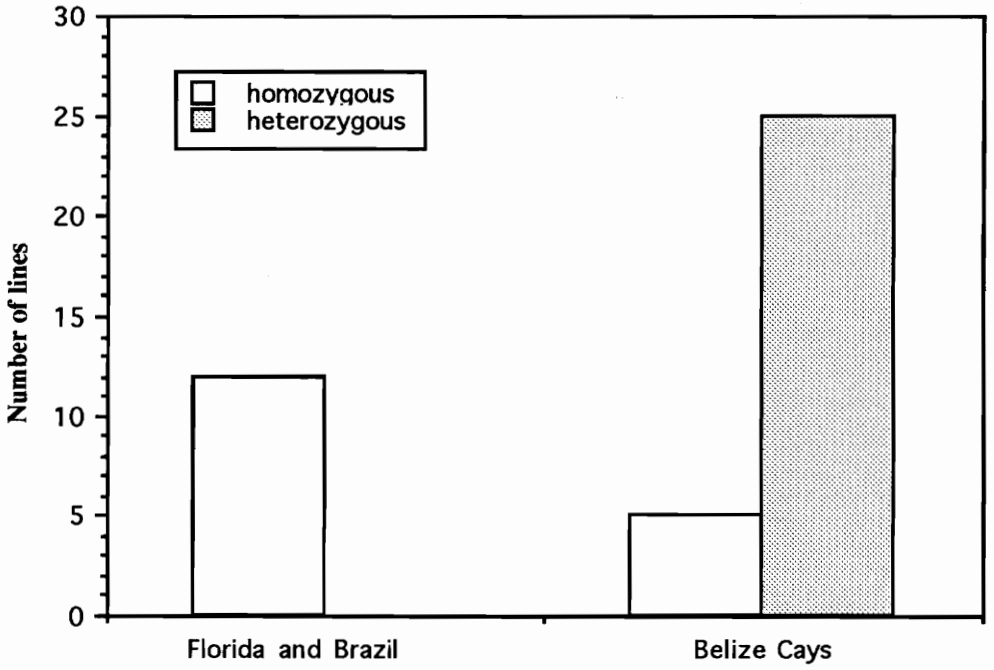

Figure 19. Total numbers of Florida and Brazil lines (combined) and Belize Cay lines scored as homozygous or heterozygous from DNA fingerprint data.

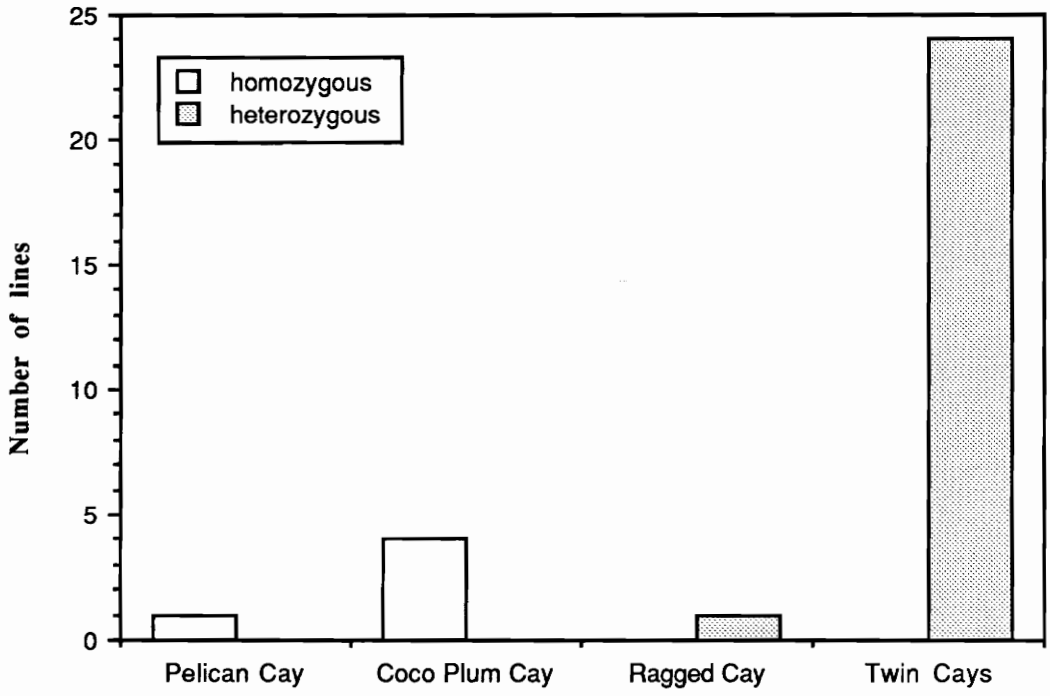

Figure 20. Total numbers of Belize Cay lines from Pelican Cay, Coco Plum Cay, Ragged Cay, and Twin Cays scored as homozygous or heterozygous from DNA fingerprint data.

OFFSPRING DATA

The total numbers of hermaphrodite and male offspring raised from eggs collected in the laboratory differed between the Florida and Brazil hermaphrodite parents (combined) and the Belize Cay hermaphrodite parents (Figure 21). The proportion of male offspring produced by Belize Cay hermaphrodites was significantly greater than the proportion of male offspring produced by the Florida and Brazil hermaphrodites ($p<0.0001$, Fisher's Exact Test).

The proportion of male offspring produced by Florida and Brazil hermaphrodites differed between lines (Figure 22). The proportion of male offspring produced from Florida and Brazil hermaphrodites ranged from approximately 0 to 0.63 (Table 8). A shorter-term laboratory line from No Name Key, FL produced the highest proportion of male offspring, while a longer-term laboratory line from Miami produced the lowest proportion of male offspring. Using the total numbers of male and hermaphrodite offspring produced per line and pooling the 2 Marco Island/Rookery Bay lines (so no more than 20% of the cells had expected values less than 5), a Chi square Test of Homogeneity indicated a significant difference between the lines ($p<0.001$, df = 7, Chi square = 26.5).

The proportion of male offspring produced by Belize Cay hermaphrodites also differed between lines (Figure 23). The heterozygous individuals from Twin Cays seem to be producing more male offspring than homozygous individuals from Coco Plum Cay and Pelican Cay. When comparing lines from Coco Plum Cay and Twin Cays, the 2 groups produced a significantly different proportion of male offspring ($p=0.02$, Mann Whitney U test statistic = 12.5). Other comparisons were not made due to small sample sizes. The total numbers of male and hermaphrodite offspring produced by Belize Cay hermaphrodites also differed between islands (Table 9). Using the total numbers of male and hermaphrodite offspring produced per island, a Chi square Test of Homogeneity indicated a significant

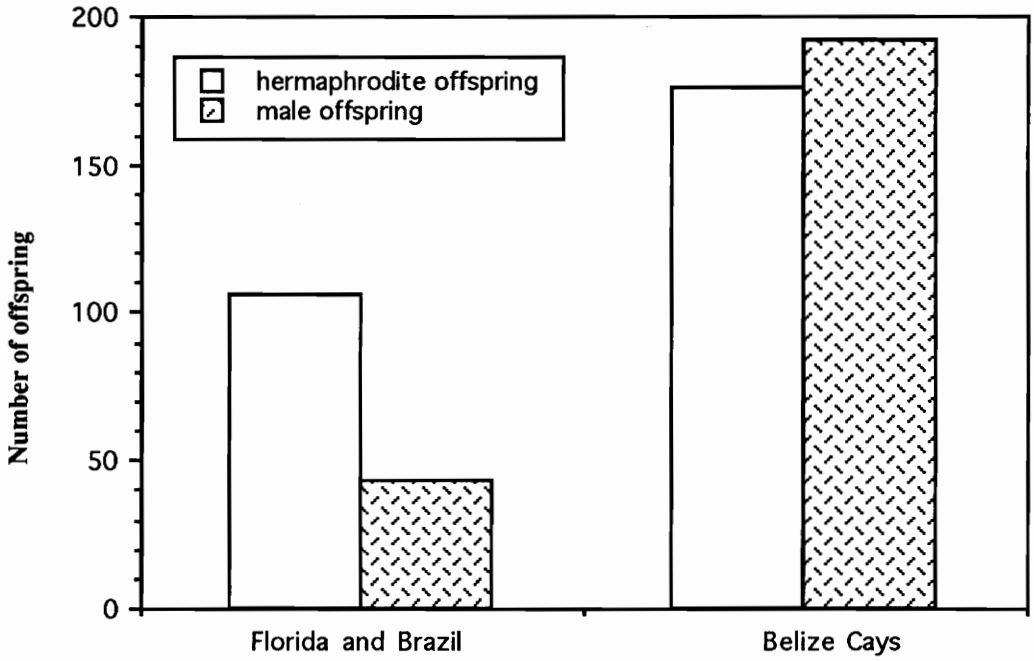

Figure 21. Total numbers of hermaphrodite and male offspring raised in the laboratory from Florida and Brazil hermaphrodites (combined) and from Belize Cay hermaphrodites. Parents included were only those analyzed by DNA fingerprinting.

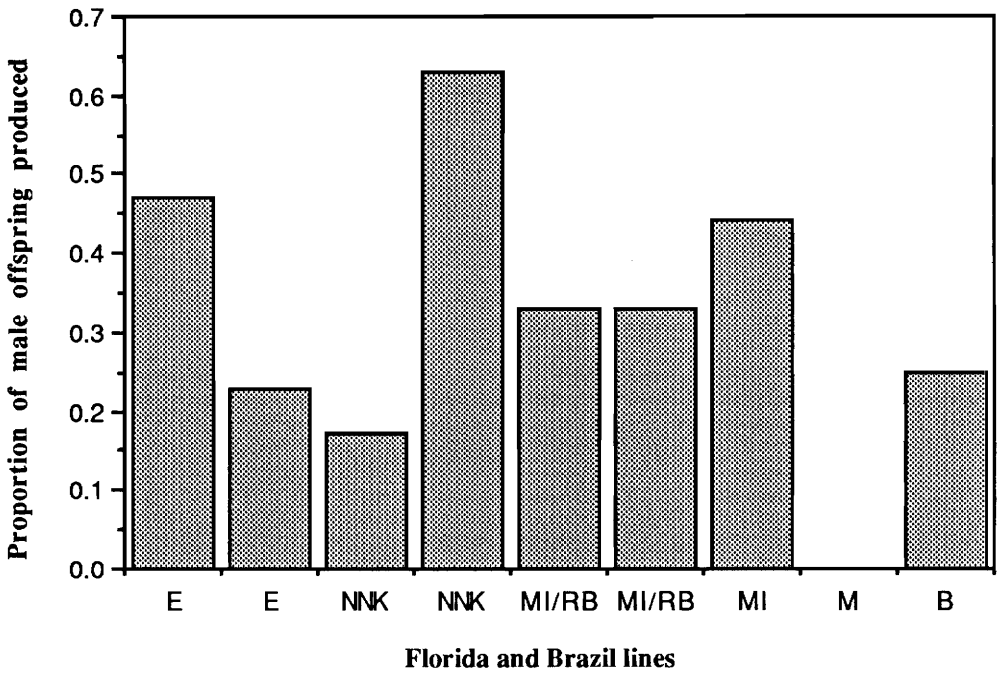

Figure 22. Variation among laboratory-maintained Florida and Brazil lines in the proportion of male offspring produced in the laboratory. The total proportion of male offspring produced per line is shown. Geographic locations of lab line progenitors: E = Everglades N.P., FL; NNK = No Name Key, FL; MI/RB = Marco Island/Rookery Bay, FL; MI = Marco Island, FL; M = Miami, FL; B = Brazil.

Table 8. Total numbers of male and hermaphrodite offspring produced in the laboratory per line by Florida and Brazil hermaphrodites. Geographic locations of laboratory line progenitors: E = Everglades N.P., FL; NNK = No Name Key, FL; MI/RB = Marco Island/Rookery Bay, FL; MI = Marco Island, FL; M = Miami, FL; B = Brazil. Sample size indicates the number of fish within each line from whom offspring were raised. Proportion of male offspring = # male offspring / total # offspring. (See Appendix B.1 for raw data from all 26 fish.)

Florida and Brazil lines	sample size	male offspring	herm. offspring	total offspring	proportion of male offspring
E - 1	1	7	8	15	0.47
E - 2	2	3	19	22	0.14
NNK - 1	2	4	19	23	0.17
NNK - 2	3	12	7	19	0.63
MI/RB - 1	2	5	10	15	0.33
MI/RB - 2	4	4	8	12	0.33
MI	3	4	5	9	0.44
M	3	0	18	18	<0.055
B	6	4	12	16	0.25
TOTAL - ALL	26	43	106	149	0.29

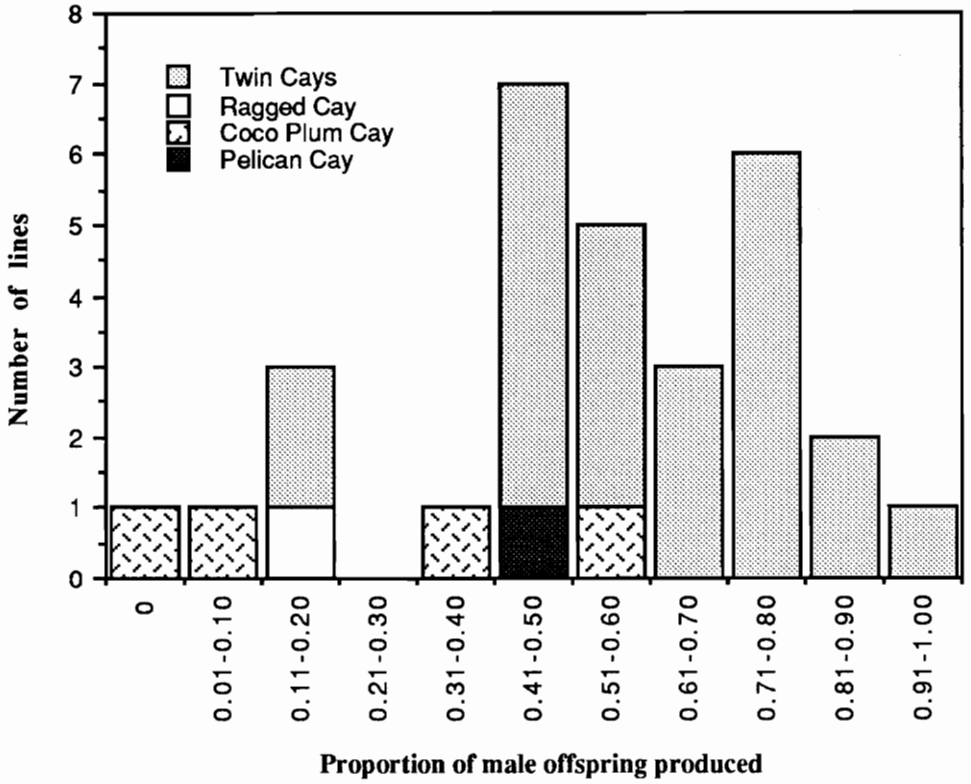

Figure 23. Distribution of the proportion of male offspring produced in the laboratory from field-caught hermaphrodites collected on the Belize islands of Twin Cays, Ragged Cay, Coco Plum Cay, and Pelican Cay.

Table 9. Total numbers of male and hermaphrodite offspring produced in the laboratory per island by Belize hermaphrodites from Pelican Cay, Ragged Cay, Coco Plum Cay, and Twin Cays. Sample size indicates the number of different fish from each island from whom offspring were raised. Proportion of male offspring = # male offspring / total # offspring. (See Appendix B.2 for raw data from all 30 fish.)

Island	sample size	male offspring	herm. offspring	total offspring	proportion of male offspring
Pelican Cay	1	7	9	16	0.44
Ragged Cay	1	2	15	17	0.12
Coco Plum Cay	4	12	43	55	0.22
Twin Cays	24	171	109	280	0.61
TOTAL - ALL	30	192	176	368	0.52

difference between islands ($p<0.001$,df=3, Chi square=40.8). The proportion of male offspring produced by Belize Cay hermaphrodites was not correlated with either PSB values determined by DNA fingerprinting (Pearson's r=0.29, p=0.12; Spearman's rho=0.21, p=0.26) or with APD values determined by DNA fingerprinting (Pearson's r=0.25, p=0.17; Spearman's rho=0.20, p=0.30) (Figure 24). These data indicate that the proportion of male offspring produced by Belize Cay hermaphrodites was not correlated with the heterozygosity level of the parent.

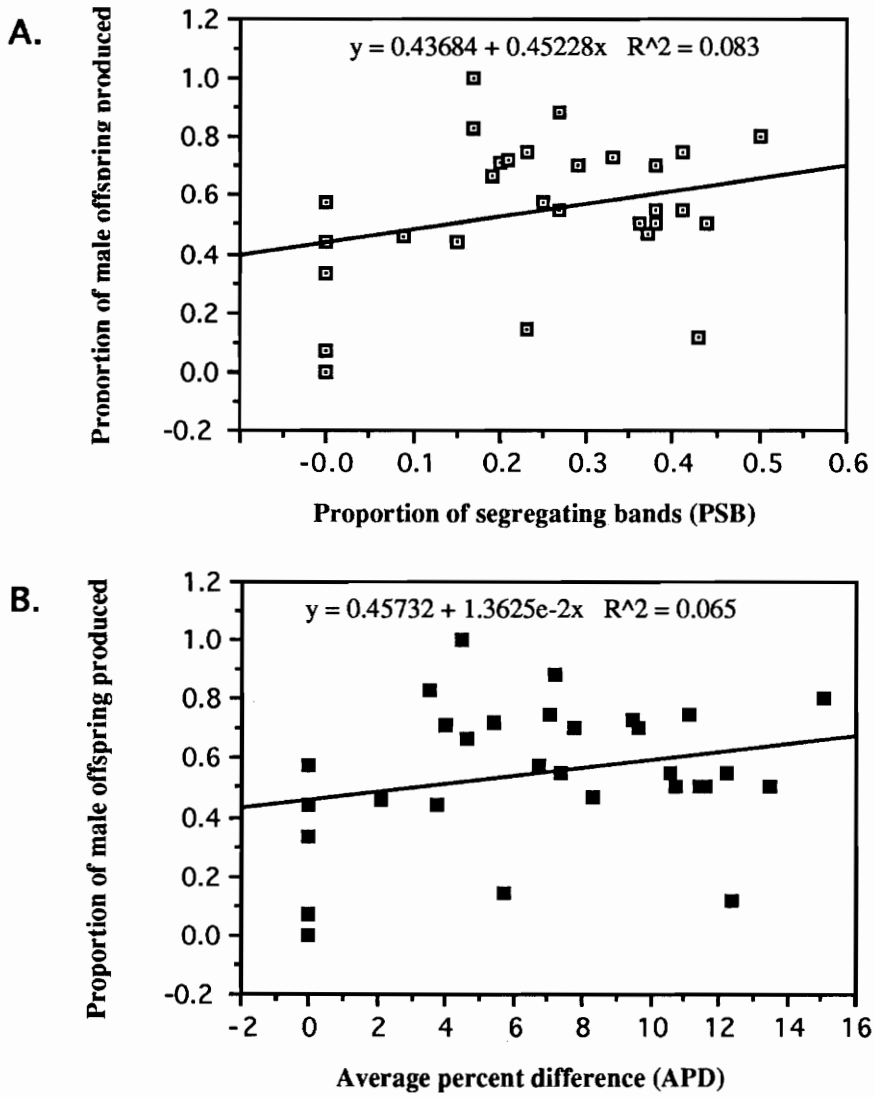

Figure 24. Scatterplots of the proportion of male offspring produced in the laboratory by Belize Cay hermaphrodites versus the heterozygosity level of the parent determined by DNA fingerprinting. Sample size = 30 fish. A.) Proportion of male offspring produced versus the proportion of segregating bands (PSB). B.) Proportion of male offspring produced versus the average percent difference (APD) among the offspring.

DISCUSSION

Detection of outcrossing in R. marmoratus

No segregation of DNA fingerprint markers among the offspring, PSB=0 and APD=0, in 12 of 12 Florida and Brazil laboratory lines and in 5 of 30 Belize Cay lines indicates the hermaphrodite parents of these individuals are homozygous. No detectable outcrossing has occurred in these populations. These results confirm earlier studies regarding the homozygosity of clonal lines (Kallman and Harrington, 1964; Harrington and Kallman, 1968) and the clonal stability and clonal inheritance of DNA fingerprint markers (Turner et. al., 1990, 1991, 1992b). However, the detection of DNA fingerprint markers segregating among the offspring, PSB>0 and APD>0, in 25 of 30 Belize Cay lines indicates the hermaphrodite parents of these individuals are heterozygous at some VNTR loci. Males are clearly involved in the mating system in male-rich Belize Cay populations. These data are the first evidence of outcrossing in this species and illustrate the utility of DNA fingerprinting to detect this novel mating system.

The proportion of segregating bands (PSB) and the average percent difference among the progeny (APD) reflect the amount of heterozygosity detected among Belize Cay lines. On Twin Cays, where all fish collected were heterozygous, PSB values ranged from 0.09 to 0.50 (combined probe data, Table 5). At least 2 parental bands were segregating among the progeny in lines with the lowest PSB values. An average PSB value of 0.30 (Table 5) (30% of the parental bands segregating among the offspring) indicates that in most individuals, many loci are segregating. On Twin Cays, APD values ranged from 2.08% to 15.09% (combined probe data, Table 7). Although values may vary with probe used (Turner et. al., 1992b), an average APD value of 8.05% $\pm$ 0.71 (s.e.) (Table 7) (offspring are 8% dissimilar to one another) on Twin Cays between siblings is similar to the average APD of 6% between individuals within inbred colonies of naked mole

rats (Reeve et. al., 1990). In other fingerprint studies of inbred or genetically isolated populations of vertebrates, APD values between individuals ranged from 0 to 29% (Gilbert et. al., 1990, 1991; Wayne et. al., 1991). There are two possible explanations to account for the range in heterozygosity values between Twin Cay lines. Matings may have occurred at different times in the past resulting in various generations of self-fertilization before individuals were collected, or matings may have occurred between individuals of various degrees of relatedness. Currently, these 2 explanations are not distinguishable. If the clonal population structure on Twin Cays is similar to Florida populations in which clones are divergent from one another and there are approximately 1.4 individuals per clone (Turner et. al., 1992b), then a mating would most likely involve two divergent individuals resulting in offspring heterozygous at all VNTR loci. If fish on Twin Cays are more related to one another than expected (this is currently being investigated), some matings may involve closely related individuals.

The presence of males in natural populations seems indicative of outcrossing. Although all Florida lines in this study were scored as homozygous, rare males have been collected in southeastern and southwestern Florida (Davis et. al., 1990). Theoretically, these rare males could be involved in the mating system but outcrossing has not been detected because of the low frequency of outcrossing events and/or because self-fertilization in heterozygous lines has restored homozygosity. While some of the laboratory lines used in this study originated from fish collected before 1989, other laboratory lines were younger and some parents included in this study were F_1 offspring of the original field-caught hermaphrodite. In these cases, heterozygosity would not have been greatly reduced. In Belize Cay populations, no males were collected and no outcrossing was detected on Coco Plum Cay (4 fish), but males were collected and outcrossing was detected on Twin Cays (24 fish). A sample size of one hermaphrodite parent and its offspring from Pelican Cay and from

Ragged Cay precludes any conclusions regarding outcrossing on these islands. However, while no males were collected on either island (0 out of 3 fish and 0 out of 40 fish for Pelican Cay and Ragged Cay respectively) (Table 1), the Pelican Cay individual was homozygous while the Ragged Cay individual was heterozygous. This suggests that there may be a high rate of migration between islands or males were missed on Ragged Cay due to sampling error. A heterozygous individual from nearby Twin Cays may have migrated to Ragged Cay. Males may have been present at one time on Ragged Cay but were no longer present at the time of sampling. Males may have been present but at extremely low frequencies or missed due to the difficulty in distinguishing males from hermaphrodites in the field. Many of these 1991 field-caught individuals from Ragged Cay were later distinguishable as males in the laboratory (personal observation). These fish were either always males (but too dark to detect orange pigmentation) or became males later. Larger numbers of fish should be collected from Ragged Cay to determine if males are present and to determine the frequency of heterozygous hermaphrodites on the island.

One of the assumptions of this study was that mutation was negligible and not confused with segregation of heterozygous VNTR loci resulting from outcrossing. The Florida and Brazil laboratory lines along with some of the original Belize Cay parents were included in this study as controls for mutational events causing fingerprint variation among the progeny. Mutation was detected among the progeny as the appearance of novel fragments (not visible in the parent). Estimated mutation rates of 3.0-6.7 x 10^{-3} (Table 2) among laboratory line progeny and among the progeny of Belize Cay hermaphrodites were comparable to estimated mutation rates of 1-4 x 10^{-3} per locus per generation in other organisms using $(CAC)_5$ and other probes (Jeffreys et. al., 1985; Burke and Bruford, 1987; Schafer et. al., 1988). Only when apparent segregation results from extremely low levels of heterozygosity could mutation be confused as

segregation. However, most Belize Cay lines were scored as heterozygous with more than 2 parental bands segregating among the progeny. This indicates that mutation shouldn't interfere with the detection of segregation even in Belize Cay lines without the parent present. A fairly high estimated mutation rate of 1.0×10^{-2} resulted from new parental bands (not in offspring) in Florida lines with $(GGCAGG)_4$ (Table 2). The GGCAGG motif found in mouse minisatellites apparently contributes to germline instability; estimated mutation rates using this motif as a probe in mice can be as high as 3.3 to 8.8×10^{-2} per locus per gamete (Mitani et. al., 1990). However, new bands in the parent would result from somatic mutations, not germline mutations, and were not of concern regarding the detection of segregation among the offspring. Thus, homozygosity among 12 Florida and Brazil lines and 5 Belize Cay lines, low mutation rates at VNTR loci, PSB and APD values in Belize Cay lines significantly greater than zero, and the segregation of at least 2 loci in Belize Cay heterozygous lines demonstrate that the heterozygosity detected in this study resulted from sexual mating and not from high levels of mutation.

Male induction in R. marmoratus

For outcrossing to evolve from a selfing population, two mutational steps are required: a female-sterility mutation (creating males) and a male-sterility mutation (creating females) (Lloyd, 1975; Charlesworth and Charlesworth, 1987). Since it is unlikely that both mutations will occur simultaneously, a transitional phase should occur. Androdioecy (the presence of males and hermaphrodites in a population) would represent an intermediate phase between selfing and outcrossing, but it is not thought to be commonly involved because of its rarity in natural populations (Charlesworth, 1984). Androdioecy has only been documented in a few species of animals and plants. These include barnacles of the order *Thoracia* (Crisp, 1983), the clam shrimp, *Eulimnadia texana* (Sassaman and Weeks,

1993), and the flowering plant, *Datisca glomerata* (Liston et. al., 1990). A female-sterility mutation can't be established in selfing populations unless male fertility is more than twice the male fertility of hermaphrodites (Lloyd, 1975, Charlesworth and Charlesworth, 1987). Thus, a female-sterility mutation is not likely to invade selfing populations (Charlesworth, 1984). Contradictory to theory, functional androdioecy exists in *R. marmoratus* even though hermaphrodites self-fertilize.

Males have been collected on the Belize Cays since sampling began in 1988. While no males were collected on the islands of Coco Plum Cay, Ragged Cay, or Pelican Cay in 1991, males were in high abundance on Twin Cays from 1988 to 1992 (Table 1) and have been collected from other islands. These islands include Blue Ground Range (1 male out of 3 fish) and Tobacco Range (1 out of 19 fish) in 1989 (Turner et. al., 1992a), and Douglas Cay (4 out of 63 fish) and Man-of-War Cay (7 out of 42 fish) in 1992 (personal observation).

Are males produced by a female sterility-mutation that has been established in some populations? In *R. marmoratus*, hermaphrodites collected where no males occur can still produce male offspring in the lab. Rather, male induction appears to be under environmental influence. In the laboratory, Harrington (1967, 1968, 1971, 1975) produced males by rearing eggs at low temperatures (18-20°C), by exposing embryos or juveniles to high temperatures (30°C), and by exposing hermaphrodites to high temperatures within the first six months of life and later exposing them to short-day season treatments. Such low temperatures are probably not ecologically relevant on the Belize Cays, but males could possibly be due to high-temperature induced sex succession (Turner et. al., 1992a). Why are males more common on some Belize islands than on others? The prevalence of males in some populations is not understood. It is reasonable to assume that environmental factors may vary between Florida and Belize, but probably not between the Belize islands.

Is a genetic component also involved in male induction? Do some individuals have lower threshold levels of induction? Harrington (1975) found that the efficiency and threshold of induction in the lab varied between clonal lines. Offspring used in this study to detect outcrossing were also scored as males or hermaphrodites to study the sex ratios produced from different fish. There were significant differences in the proportion of male offspring produced in the lab between lines from Florida and Brazil, between hermaphrodites from the 4 Belize Cays, and between Florida and Brazil hermaphrodites (combined) and the Belize Cay hermaphrodites. However, there was no correlation of heterozygosity level of Belize Cay hermaphrodites with the proportion of male offspring produced (Fig. 24). This suggests that heterozygosity per se apparently has no discernible influence on sex ratio. While neither parents nor offspring were kept at constant temperatures during rearing, fish were probably randomly located in the lab and exposed to similar fluctuations. However, it is possible that other conditions biased the sex ratio. Offspring scored were those that had survived (many died or were eaten); this should not bias the sex ratio unless one sex has a higher mortality rate than the other. Not all offspring were raised individually; some were raised with siblings. Though crowding probably affects size, it is not known whether the sex ratio will be affected. While the laboratory conditions may not be indicative of natural conditions, these differences in sex ratios among individuals and between populations suggest a genetic component to sex induction may also exist.

Both environmental and genetic factors seem to affect sex ratios in other organisms (Conover and Heins, 1987; Naylor et. al., 1988; Janzen, 1992). Sex determination in the fish, *Menidia menidia*, seems to be controlled by an interaction between major genetic factors, polygenic factors and temperature (Lagomarsino and Conover, 1993). Sex in some populations of this species is environmentally determined, while it is genetically determined in

other populations; the differences among populations may be due to selection and adaptation to local environmental conditions (Conover et. al., 1992). The rarity of male *R. marmoratus* in Florida populations may be indicative of strictly genetic influences determining sex, but Florida fish are sensitive to temperature in the laboratory. Original studies investigating male induction (Harrington, 1967, 1968) were performed with Florida fish, and most Florida hermaphrodites in this study did produce male offspring (Table 8). So why aren't more males found in Florida populations when Florida fish can produce males? Are other environmental factors involved? Perhaps Florida fish have male induction systems with higher temperature thresholds and thus appear less sensitive to temperature, while some Belize individuals have lower temperature thresholds. Currently, the mechanisms of male induction and the prevalence of males on some Belize islands are not understood.

In the flowering plant, *Datisca glomerata*, outcrossing rates are high enough (65-92%) (Fritsch and Rieseberg, 1992) to meet the theoretical requirement (male fertility is more than twice the male fertility of hermaphrodites) for the maintenance of males (Lloyd, 1975; Charlesworth and Charlesworth, 1987) . In the clam shrimp, *Eulimnadia texana*, males are thought to be maintained when they are highly fecund and fertilize more than 2 hermaphrodites on average (Otto et. al., 1993). Outcrossing rates on Twin Cays (24/24=100%) would be high enough to meet the theoretical requirement, but the outcrossing rate may be overestimated. The actual frequency of outcrossing events is not known since some of these individuals may be offspring of the same outcrossing events. Males in the androdioecious Twin Cays population don't appear to be maintained at equal frequencies from year to year. The proportion of males on Twin Cays has declined significantly from 1988 to 1992, while sample sizes have increased (Table 1) ($p<0.001$, $df=3$, Chi square = 31.3). This may be an indication that the situation on Twin Cays is temporary and not a transition to complete outcrossing, but

even a low frequency of males may be enough to maintain the androdioecious system.

Self-fertilization in R. marmoratus

For outcrossing to occur in a selfing population, not only do highly fecund males need to be present, but a mutation must also occur to reduce the efficiency of self-fertilization of hermaphrodites (a male-sterility mutation) (Charlesworth, 1984; Charlesworth and Charlesworth, 1987). For this allele to spread in a population, outcrossed progeny must be at least twice as fit as selfed progeny; thus, theory predicts that selfing populations aren't likely to revert to outcrossing (Lande and Schemske, 1985; Charlesworth et. al., 1990). Contradictory to theory, outcrossing does exist in *R. marmoratus* even though self-fertilization appears efficient.

Do hermaphrodites become females by inheriting a male-sterility mutation? This implies a permanent cessation of male functioning. The 25 Twin Cays heterozygous hermaphrodites all produced fertile eggs in the laboratory. Rather, a temporary reduction in selfing seems more plausible. Hermaphrodites of *R. marmoratus* must somehow reduce the efficiency of self-fertilization and act as females by suppressing the testicular portion of the ovotestis to lay infertile eggs. Harrington (1971) postulated that this response may be seasonal, age-dependent, environmental, or due to male influence (visual or pheromonal). Kristensen (1970) claimed that hermaphrodites laid eggs in various developmental stages (as described by Harrington, 1963) when males were absent but laid eggs showing no signs of development when males were present. However, as pointed out by Turner et. al. (1992a), Kristensen used no genetic data to determine paternities and developmental differences could account for his observations. If hermaphrodites can somehow lay infertile eggs only when males are present and fertilization is ensured, this would be a way to avoid reproductive failure which would occur if infertile eggs were laid in the absence of males. There

is some evidence that temperature may affect fertility of hermaphrodites, but lower fertility was observed when hermaphrodites were exposed to low temperatures (probably irrelevant to the Belize Cay hermaphrodites) (Harrington, 1971). Currently, the mechanisms involved in the physiological response of hermaphrodites to reduce self-fertilization are not understood.

Implications of outcrossing in R. marmoratus

The mating system of *R. marmoratus* on Twin Cays appears complex. Both males and hermaphrodites must be present, hermaphrodites must lay infertile eggs, and males must fertilize these eggs externally. There is no known mechanism for internal cross-fertilization (Turner et. al., 1992a), and hermaphrodites cannot cross-fertilize one another. Though spawning embraces have been observed, hermaphrodites are generally aggressive and often kill males (Turner et. al., 1992a). Thus, hermaphrodites may have to also respond behaviorally to allow males to fertilize eggs.

Outcrossing does occur in male-rich Belize Cay populations of *R. marmoratus* despite theoretical predictions against it and despite the costs involved. Sexual reproduction is expensive. Individuals can no longer transmit gene combinations efficiently: any particular gene is transmitted to only half the offspring. This represents the cost of sex (Maynard Smith, 1978) or the cost of meiosis (Williams, 1975). Also, males must be produced and mating must occur. A reduction in fitness (outbreeding depression) can also occur due to the alteration by recombination of non-random adaptive gene associations (Charlesworth and Charlesworth, 1978). In *R. marmoratus*, hermaphrodites may lose resources needlessly if infertile eggs are laid and not fertilized by males. If outcrossing becomes obligatory, the ability to colonize new habitats is diminished. So how is sex accomplished or maintained despite these costs? This represents the dilemma or paradox of sex and has been the subject of much debate. The cost of outcrossing must be balanced by the cost of selfing,

perhaps by the reduction in fitness from inbreeding depression (Charlesworth, 1980). However, outcrossing seems unlikely to evolve only to avoid inbreeding depression, though it may be important in the evolutionary process (Charlesworth and Charlesworth, 1987). In selfing populations, such as *R. marmoratus*, high levels of inbreeding depression aren't expected because of the elimination of recessive deleterious alleles (Shields, 1982). Inbreeding depression, though, can also result from the lack of heterozygotes of superior fitness to homozygotes (Charlesworth and Charlesworth, 1987). Generally, there is a positive relationship between heterozygosity and fitness (Allendorf and Leary, 1986), but this may not be a general relationship in all species (Booth et. al., 1990; Whitlock, 1993).

If the heterozygous progeny created by outcrossing are not of high fitness advantage, outcrossing may be detrimental to *R. marmoratus*. In contrast, if heterozygous progeny are of high fitness advantage, outcrossing will be beneficial. Since self-fertilization has not been lost, a heterozygous individual can now colonize new habitats and produce variable offspring. These offspring will be advantageous under changing environmental conditions, especially when populations are dense and resources are limited (Bell, 1982). Thus, on Twin Cays, the benefits of sexual reproduction may outweigh the costs involved. The evolutionary potential of the clonal fish, *Rivulus marmoratus*, may need to be re-evaluated if sexual reproduction is a regular part of the breeding biology on Twin Cays and in other populations.

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Appendix A.1. Raw data of artifact bands (novel bands) for Florida and Brazil lines. Lines included were only those in which the DNA of both parent and offspring were run on the same fingerprint gel. Bands in the offspring not in the parent are indicated as well as bands in the parent but not in the offspring. Sample size indicates the number of offspring or parents included on the fingerprint gel.

(CAC)5	OFFSPRING			PARENT		
Line	sample size	# novel bands	total # bands	sample size	# novel bands	total # bands
E125	9		171	1		19
E126	15		315	3		63
NNK	22		330	7		105
NNK3	4		68	1		17
CB	15		315	4		84
TOTAL	65	0	1199	16	0	288

(GGCAGG)4	OFFSPRING			PARENT		
Line	sample size	# novel bands	total # bands	sample size	# novel bands	total # bands
269	12	12	288	4	4	96
76	11		253	3	1	69
MI1-1	5		135	1		27
MI1-2	4		52	1		23
MI1-3	2		92	1		26
E126	15		405	3		81
NNK	22		484	7		154
NNK3	4		80	1		20
TOTAL	75	12	1789	21	5	496

Appendix A.2. Raw data of artifact bands (novel bands) for Belize lines. Lines included were only those in which the DNA of both parent and offspring were run on the same fingerprint gel. Bands in the offspring not in the parent are indicated as well as bands in the parent but not in the offspring. Sample size indicates the number of offspring or parents included on the fingerprint gel.

(CAC)5	OFFSPRING			PARENT		
Fish ID	sample size	# novel bands	total # bands	sample size	# novel bands	total # bands
Bel 14-91	6		79	1		14
Bel 26-91	5		110	1		25
Bel 27-91	5		107	1		24
Bel 33-91	5		152	1		32
Bel 45-91	5		78	1		16
Bel 54-91	5	2	137	1		28
TOTAL	31	2	663	6	0	139

(GGCAGG)4	OFFSPRING			PARENT		
Line	sample size	# novel bands	total # bands	sample size	# novel bands	total # bands
Bel 4-91	6		127	1		24
Bel 14-91	6		162	1		30
Bel 32-91	5		57	1		12
Bel 43-91	5		70	1		15
Bel 45-91	5	1	69	1		15
Bel 48-91	6		144	1		24
Bel 54-91	5		88	1		18
Bel 67-91	5		74	1		18
Bel 99-91	6	4	186	1		31
Bel 100-91	5	1	80	1		16
TOTAL	54	6	1057	10	0	203

Appendix B.1. Numbers of male and hermaphrodite offspring produced in the laboratory by Florida and Brazil hermaphrodites.

Florida and Brazil lines	# male offspring	# herm. offspring	proportion of male offspring	line totals # male/ total
#1 - Clone M				0/18=<0.055
128-91	0	7	0	
129-91	0	9	0	
131-91	0	2	0	
#2 - 208				5/15=0.33
117-91	3	5	0.38	
125-91	2	5	0.29	
#3 - 269				4/12=0.33
120-91	1	2	0.33	
121-91	0	2	0	
122-91	3	3	0.5	
123-91	0	1	0	
#4 - NNK				4/23=0.17
133-91	1	6	0.14	
134-91	0	10	0	
135-91	3	3	0.5	
#5 - E125				7/15=0.47
1	7	8	0.47	
#6 - E126				3/22=0.14
1	0	6	0	
2	3	13	0.23	
#8 - MI1				4/9=0.44
1	2	2	0.5	
2	2	2	0.5	
3	0	1	0	
#9 - NNK3				12/19=0.63
1	7	0	1	
2	5	7	0.42	
#12 - RO				4/16=0.25
119-91	0	2	0	
137-91	0	3	0	
bjt 131	0	1	0	
bjt 132	0	2	0	
bjt 133	2	2	0.5	
bjt 134	2	2	0.5	
TOTAL - all	**43**	**106**	**0.29**	

Appendix B.2. Numbers of male and hermaphrodite offspring produced in the laboratory by Belize hermaphrodites (n=30 fish).

Belize islands	# male offspring	# herm. offspring	proportion of male offspring	island totals #male/ total
Pelican Cay				
Bel 40-91	7	9	0.44	2/15=0.12
Ragged Cay				
Bel 2-91	2	15	0.12	7/9= 044
Coco Plum Cay				
Bel 48-91	7	5	0.58	
Bel 50-91	4	8	0.33	
Bel 99-91	1	14	0.07	
Bel 100-91	0	16	0.00	
CP total	12	43		12/55=0.22
Twin Cays				
Bel 4-91	7	3	0.70	
Bel 5-91	4	5	0.44	
Bel 6-91	7	3	0.70	
Bel 11-91	4	4	0.50	
Bel 14-91	7	1	0.88	
Bel 17-91	11	9	0.55	
Bel 18-91	1	6	0.14	
Bel 19-91	7	5	0.58	
Bel 26-91	6	5	0.55	
Bel 27-91	8	3	0.73	
Bel 28-91	5	5	0.50	
Bel 29-91	18	7	0.72	
Bel 30-91	7	7	0.50	
Bel 32-91	7	0	1.00	
Bel 33-91	8	4	0.67	
Bel 34-91	5	2	0.71	
Bel 35-91	6	5	0.55	
Bel 39-91	7	8	0.47	
Bel 43-91	6	6	0.50	
Bel 45-91	10	2	0.83	
Bel 54-91	11	13	0.46	
Bel 61-91	6	2	0.75	
Bel 67-91	4	1	0.80	
Bel 80-91	9	3	0.75	
TC total	171	109		171/280 =0.61
TOTAL - all	**192**	**176**	**0.52**	

Curriculum Vita
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ACADEMIC DEGREES

Master of Science	Virginia Polytechnic Institute and State University Blacksburg, VA GPA: 3.58 / 4.0	Biology	Sept.1993

Thesis: *Using molecular genetic techniques to detect outcrossing in natural populations of a self-fertilizing fish.*

Advisor: Bruce J. Turner

Bachelor of Science	Bowling Green State University Bowling Green, OH GPA: 3.82 / 4.0, Magna Cum Laude	Biology	May 1988

PROFESSIONAL EMPLOYMENT

Graduate Teaching Assistant: August 1990 - June 1993.
Biology Department (D. Stetler), VPI & SU, Blacksburg, VA.
Courses taught: Molecular Biology Laboratory
Principles of Biology Laboratory (majors)
General Biology Laboratory (non-majors)
Human Anatomy and Physiology Laboratory

Field Assistant: May - August 1990.
Biology Department (C. Galen), BGSU, Bowling Green, OH.
Floral evolution, pollination ecology, and population genetics of alpine plants.

Graduate Teaching Assistant: August 1988 - May 1990.
Biology Department (D. Fritsch), Virginia Commonwealth University, Richmond, VA.
Courses taught: General Biology Laboratory (non-majors)

Undergraduate Research Assistant: August 1987 - May 1988.
Biology Department (R. Woodruff), BGSU, Bowling Green, OH.
Genetic studies involving mutagenesis in *Drosophila melanogaster.*

Field Assistant: May - August 1987.
Biology Department (C. Galen), BGSU, Bowling Green, OH.
Floral evolution and pollination ecology of alpine plants.

Undergraduate Research Assistant: August 1986 - May 1987.
Biology Department (C. Galen), BGSU, Bowling Green, OH.
Floral evolution and pollination ecology of alpine plants.

SKILLS

Fish collection and maintenance
Experience with the field collection and laboratory rearing of *Rivulus marmoratus*.
DNA fingerprinting
Experience with DNA fingerprinting techniques: DNA isolation, quantification, digestion, electrophoresis, hybridization, autoradiography.
Experience with DNA fingerprint analysis.
Computer
Computer experience with Macintosh systems and word processing, statistics, drafting, and graphics software.

ACTIVITIES

1991 - 1993 Association of Biology Graduate Students
Election Committee: 1991-1992
Evolution Seminar Series Committee: Spring 1992

AWARDS

Graduate Research Poster Symposium, Second Place, VPI & SU, March, 1993.

GRANT SUPPORT

1992 American Museum of Natural History, Lerner Gray Grants for Marine Research. Proposal funded for $400.

1992 VPI & SU Biology Department matching grant, $400.

1992 Sigma Xi Grant-in-Aid of Research. Proposal funded for $350.

1992 VPI & SU Biology Department matching grant, $350.

PUBLIC PRESENTATIONS

"The role of males in natural populations of a self-fertilizing hermaphroditic fish." Seminar presented in the Evolution Seminar Series, Biology Department, VPI & SU, January 1993.

"Patch selection by bumblebees foraging in natural populations of *Polemonium viscosum* and other alpine plants." Seminar presented in the Ecolunch Seminar Series, Biology Department, Bowling Green State University, April 1988.

ABSTRACTS

Lubinski, B.A. and B.J. Turner. 1993. First evidence of outcrossing in *Rivulus marmoratus*, a selfing hermaphroditic fish. Graduate Research Poster Symposium, VPI & SU, March 1993.

Lubinski, B.A. and B.J. Turner. 1992. First evidence of recombination in *Rivulus marmoratus*, a clonal selfing hermaphrodite. Poster session, American Genetics Association Symposium, The Evolution of Sex, Blacksburg, VA, July 1992.

Turner, B.J., B.A. Lubinski, W.D. Davis, and D.S. Taylor. 1992. *Rivulus marmoratus*: the challenges of a selfing hermaphroditic vertebrate. Poster session, American Genetics Association Symposium, The Evolution of Sex, Blacksburg, VA, July 1992.

MANUSCRIPTS

Lubinski B.A., W.P. Davis, D.S. Taylor, and B.J. Turner. *In preparation.* Outcrossing in a male-rich population of a selfing hermaphroditic fish detected by DNA fingerprinting.